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 NEWS  3  Feb 06   Engineering Information Encompass files have new names
 NEWS  4  Feb 16   TOXLINE no longer being updated
 NEWS  5  Apr 23   Search Derwent WPINDEX by chemical structure
 NEWS  6  Apr 23   PRE-1967 REFERENCES NOW SEARCHABLE IN CAPLUS AND CA
 NEWS  7  May 07   DGENE Reload

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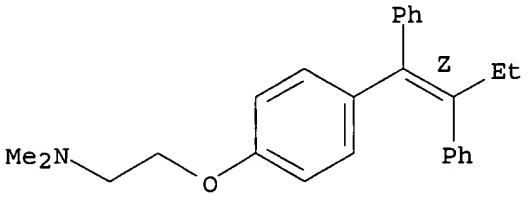
CA INDEXING COPYRIGHT (C) 2001 AMERICAN CHEMICAL SOCIETY (ACS)

=> s endothelium (w) damage

L1          289 ENDOTHELIUM (W) DAMAGE
```

L1 ANSWER 1 OF 1 REGISTRY COPYRIGHT 2003 ACS on STN
RN 10540-29-1 REGISTRY
CN Ethanamine, 2-[4-[(1Z)-1,2-diphenyl-1-butenyl]phenoxy]-N,N-dimethyl- (9CI) (CA INDEX NAME)
OTHER CA INDEX NAMES:
CN Ethanamine, 2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethyl-, (Z)-
CN Ethylamine, 2-[p-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethyl-, (Z)- (8CI)
OTHER NAMES:
CN ICI 47699
CN Mammaton
CN **Tamoxifen**
CN trans-Tamoxifen
CN Z-Tamoxifen
FS STEREOSEARCH
MF C26 H29 N O
CI COM
LC STN Files: ADISNEWS, AGRICOLA, ANABSTR, AQUIRE, BEILSTEIN*, BIOBUSINESS, BIOSIS, BIOTECHNO, CA, CANCERLIT, CAPLUS, CASREACT, CBNB, CEN, CHEMCATS, CHEMINFORMRX, CHEMLIST, CIN, CSCHEM, CSNB, DDFU, DIOGENES, DRUGU, EMBASE, HSDB*, IMSCOSEARCH, IMSDRUGNEWS, IMSPATENTS, IPA, MEDLINE, MRCK*, MSDS-OHS, NIOSHTIC, PHAR, PROMT, RTECS*, SPECINFO, TOXCENTER, ULIDAT, USAN, USPAT2, USPATFULL, VETU
(*File contains numerically searchable property data)
Other Sources: EINECS**, WHO
(**Enter CHEMLIST File for up-to-date regulatory information)

Double bond geometry as shown.



PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

5205 REFERENCES IN FILE CA (1907 TO DATE)
139 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
5228 REFERENCES IN FILE CAPLUS (1907 TO DATE)

=> s endotheli?

L2 198753 ENDOTHELI?

=> s l1 or l2

L3 198753 L1 OR L2

=> s l3 (p) tamoxifen

L4 194 L3 (P) TAMOXIFEN

=> dup rem l4

PROCESSING COMPLETED FOR L4
L5 121 DUP REM L4 (73 DUPLICATES REMOVED)

=> s l5 and (vascular (w) smooth (w) muscle)

L6 19 L5 AND (VASCULAR (W) SMOOTH (W) MUSCLE)

=> dup rem l6

PROCESSING COMPLETED FOR L6
L7 19 DUP REM L6 (0 DUPLICATES REMOVED)

=> d l7 1-19 bib,kwic

L7 ANSWER 1 OF 19 USPATFULL
AN 2001:33286 USPATFULL
TI Prevention and treatment of cardiovascular pathologies with tamoxifen analogues
IN Grainger, David J., Cambridge, United Kingdom
Metcalfe, James C., Cambridge, United Kingdom
Kunz, Lawrence L., Redmond, WA, United States
Schroff, Robert W., Edmonds, WA, United States
PA NeoRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 6197789 20010306
WO 9640098 19961219
AI US 1997-973570 19971205 (8)
WO 1996-US10211 19960607
19980908 PCT 371 date
19980908 PCT 102(e) date
RLI Continuation-in-part of Ser. No. US 1995-478936, filed on 7 Jun 1995, now abandoned Continuation-in-part of Ser. No. US 1995-476735, filed on 7 Jun 1995, now patented, Pat. No. US 5595722 Continuation-in-part of Ser. No. US 1995-477393, filed on 7 Jun 1995 Continuation-in-part of Ser. No. US 1995-486334, filed on 7 Jun 1995, now patented, Pat. No. US 5770609
DT Utility
EXNAM Primary Examiner: Criares, Theodore J.
LREP Schwegman, Lundberg, Woessner & Kluth, P.A.
CLMN Number of Claims: 17
ECL Exemplary Claim: 1
DRWN 8 Drawing Figure(s); 5 Drawing Page(s)
LN.CNT 4577
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
SUMM . . . can be considered to occur in five overlapping stages such as migration, lipid accumulation, recruitment of inflammatory cells, proliferation of **vascular smooth muscle** cells, and extracellular matrix deposition. Each of these processes can be shown to occur in man and in animal models. . .
SUMM The administered compound of formula (I) can act on **vascular smooth muscle** cells (VSMC) to inhibit the pathological activity of these smooth muscle cells, can inhibit the activation of

endothelial cells, can inhibit lipid accumulation by vessels, decrease lesion formation or development, and can increase plaque stability. Preferably, the compound. . . comprises treatment of atherosclerosis, wherein the compound of formula (I), such as idoxifene or idoxifene salt, inhibits lipid accumulation by **vascular smooth muscle** cells and/or stabilizes an arterial lesion associated with atherosclerosis, i.e., increases plaque stability, to prevent rupture or growth of the lesion. As exemplified hereinbelow, orally administered **tamoxifen** significantly inhibits the formation of lipid lesions, induced by a high fat diet, in C57B16 mice and in the transgenic apo(a) mouse. The 90% reduction in lesion area and number in both of these mouse models indicates that **tamoxifen** affects the accumulation of lipid in the cells and stroma of the vessel wall. The inhibition of lipid accumulation and lesion development in these treated mice indicates that **tamoxifen** and analogs thereof, as well as compounds of formula (I), may inhibit the development of atherosclerotic lesions in humans by. . .

SUMM A further aspect of the invention is a method comprising inhibiting **vascular smooth muscle** cell proliferation associated with procedural vascular trauma due to organ transplantation, vascular surgery, angioplasty, shunt placement, stent placement or vascular. . .

SUMM Yet a further aspect of the invention provides a method comprising inhibiting non-aortal **vascular smooth muscle** cell proliferation associated with procedural vascular trauma comprising administering an effective cytostatic antiproliferative amount of tamoxifen, a structural analog thereof,. . . local, catheter or non-catheter delivery to the site of the trauma. A preferred embodiment of the invention comprises inhibiting non-aortal **vascular smooth muscle** cells in a non-coronary artery.

SUMM . . . identifying an agent which increases the level of TGF-beta, e.g., the agent is a TGF-beta activator or production stimulator. Human **vascular smooth muscle** cells (hVSMC) are cultured with an amount of the agent effective to reduce or inhibit the rate of hVSMC proliferation.. . . said hVSMC and then the rate of proliferation is determined. The method can also include the culture of rat aortic **vascular smooth muscle** cells (rVSMC) with an amount of the same agent effective to reduce or inhibit the rate of proliferation of rVSMC.. . .

SUMM . . . of formula (I) which include when R.sup.4 together pith R.sup.3 is --CH.sub.2 --CH.sub.2 -- or --S--, or R.sup.5 is OH, **tamoxifen**, and structural analogs of **tamoxifen**. These agents and compounds, including their salts and mixtures thereof, may be employed in the practice of the present invention to prevent or treat other conditions characterized by inappropriate or pathological activity of **vascular smooth muscle** cells or **endothelial** cells, excluding the inappropriate proliferation or pathological activity of neoplastic **vascular smooth muscle** cells or neoplastic **endothelial** cells. Thus, it is envisioned that the methods of the present invention preferably do not include the treatment of neoplastic. . .

SUMM The agents of the invention, which increase the level of TGF-beta, inhibit abnormal activity of **vascular smooth muscle** cells and **endothelial** cells. Preferred agents of the invention include compounds of formula (I). Preferred compounds of formula (I) include those wherein Z. . . are each CH.sub.3 or together with N are pyrrolidino, hexamethyleneimino or piperidino. These agents or compounds can include analogs of **tamoxifen** (including derivatives of TMX and derivatives of said analogs) having

equivalent bioactivity. Such analogs include idoxifene

(IDX)(E-1-[4-[2-N-pyrrolidino)ethoxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene), raloxifene, 3-iodotamoxifen, 4-iodotamoxifen,. . .

DRWD FIGS. 1 and 2 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.

DETD . . . with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a **vascular smooth muscle** cell or pericyte.

DETD The term "**tamoxifen**", as used herein, includes trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine, and the pharmaceutically acceptable salts thereof, which are capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **endothelial** cell and **vascular smooth muscle** cell activity.

Isomers and derivatives of the aforementioned chemical compound are also included within the scope of the term "**tamoxifen**" for the purposes of this disclosure.

DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta.

DETD Agents which increase the level of TGF-beta are useful for inhibiting the pathological proliferation of **vascular smooth muscle** cells or endothelial cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means. . .

DETD . . . a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells or endothelial cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke), preferably via systemic administration. The. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 1. TGF-beta is believed to contribute to the inhibitory mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels.

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. An apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 2. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. U.S.A., 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles

medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7+/-0.6 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of

nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation within ED.sub.50 (a concentration resulting in 50% inhibition) at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 1 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 2 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that are. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD . . . dosage forms involving sustained release of the TGF-beta activator or production stimulator to target cells. Preferably, the target cells are **vascular smooth muscle** cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that. . . the dosage form. Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting

vascular smooth muscle cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, formation of lipid proliferative lesions, contraction, migration or the like) but does not kill the cell and, optionally, a **vascular smooth muscle** cell binding protein. Sustained released dosage forms for systemic administration as well as for local administration are also employed in. . .

DETD . . . affect the rate and duration of the drug release required to achieve the cytostatic dosing which has been demonstrated in **vascular smooth muscle** cell tissue culture experiments. Different types of devices may require different periods of therapeutic drug release. For example, the use. . .

DETD Human **vascular smooth muscle** cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat. Medium conditioned. . .

DETD . . . is a TGF-beta activator or TGF-beta production stimulator, an agent or mixture of agents is first tested on rat aortic **vascular smooth muscle** cells (rVSMCs) for their ability to stimulate the production of active TGF-.beta. in the culture medium as originally described for. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation Cell culture, DNA synthesis assay and cell counting.

DETD Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainge. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+100% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time

of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614+/-1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-beta mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. **Tamoxifen** (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were

purchased from Flow Laboratories. 6-[.sup.3. . . factor and insulin-like growth factor 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Antiotensin II and **endothelin** 1 were obtained from Sigma and dissolved in sterile MilliQ water. TGF-beta (0.5 .mu.g, lyophilized solid) was purchased from Peninsula,. . .

DETD TABLE 8

Mitogenic indices of human serum and plasma on human **vascular smooth muscle** cells

Donor	Mitogenic index Serum	Plasma
B	45	0.7
H	52	1.4
C	60	0.9
D	65	1.0
A	83	1.2

DMEM containing 5% serum or 20%. . .

L7 ANSWER 2 OF 19 CAPLUS COPYRIGHT 2001 ACS
AN 2000:608566 CAPLUS
DN 133:172188
TI Methods to reduce the sensitivity of endothelially-compromised **vascular smooth muscle**
IN Lamb, Fred S.
PA University of Iowa Research Foundation, USA
SO PCT Int. Appl., 34 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2000050023	A2	20000831	WO 2000-US4892	20000226

W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

PRAI US 1999-121727 P 19990226
OS MARPAT 133:172188
TI Methods to reduce the sensitivity of endothelially-compromised **vascular smooth muscle**
AB The present invention discloses materials and methods useful to treat sensitivity of endothelially-compromised **vascular smooth muscle**. In one embodiment, CLC3 blockers, particularly compds. of formula I are used to treat sensitivity.
ST **vascular smooth muscle endothelial** damage treatment **tamoxifen**; CLC3 blocker **vascular smooth muscle endothelial** damage
IT Chloride channel
RL: BPR (Biological process); BIOL (Biological study); PROC (Process)
(CLC-3; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)
IT Ion channel blockers
(chloride, CLC-3; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)
IT Artery, disease
(coronary, restenosis, agents for treatment of; methods to reduce

sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT Artery, disease
(coronary, vascular **endothelial** damage in, treatment of; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT Blood vessel, disease
(diabetic angiopathy, treatment of; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT Blood vessel, disease
(**endothelium**, injury; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT Antidiabetic agents
Antihypertensives
Drug interactions
Vasodilators
(methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT Blood vessel
(smooth muscle; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT Hypertension
Surgery
(vascular **endothelial** damage from, treatment of; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT 10540-29-1, **Tamoxifen**
RL: BAC (Biological activity or effector, except adverse); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

IT 51-41-2, Norepinephrine 7447-40-7, Potassium chloride (KCl), biological studies
RL: BAC (Biological activity or effector, except adverse); BIOL (Biological study)
(vasoconstriction after **endothelial** damage from, normalization of; methods to reduce sensitivity of **endothelially**-compromised **vascular smooth muscle** using CLC3 chloride channel blockers such as **tamoxifen** in relation to use of other agents)

L7 ANSWER 3 OF 19 USPATFULL
AN 2000:174716 USPATFULL
TI Prevention and treatment of pathologies associated with abnormally proliferative smooth muscle cells
IN Grainger, David J., Cambridge, United Kingdom
Metcalfe, James C., Cambridge, United Kingdom
Weissberg, Peter L., Cambridge, United Kingdom
PA NeoRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 6166090 20001226
AI US 1997-965589 19971106 (8)

RLI Continuation of Ser. No. US 1994-242161, filed on 12 May 1994, now patented, Pat. No. US 5847007 which is a continuation-in-part of Ser. No. US 1993-61714, filed on 13 May 1993, now abandoned
DT Utility
EXNAM Primary Examiner: Henley, Jr., Raymond
LREP Schwegman, Lundberg, Woessner & Kluth, P.A.
CLMN Number of Claims: 14
ECL Exemplary Claim: 1
DRWN 2 Drawing Figure(s); 2 Drawing Page(s)
LN.CNT 2490
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB . . . dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation and/or migration of **vascular smooth muscle** cells over time. Further provided is a method for determining TGF-beta in vitro, thereby identifying a patient at risk for. . .
SUMM . . . to the prevention and treatment of conditions characterized by abnormal smooth muscle cell proliferation. More specifically, mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation and agents that impact those mechanisms are discussed.
SUMM . . . cell proliferation. It would be highly advantageous to develop new compositions or methods for inhibiting stenosis due to proliferation of **vascular smooth muscle** cells following, for example, traumatic injury to vessels rendered during vascular surgery.
SUMM . . . a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .
DRWD FIGS. 1 and 2 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.
DETD . . . with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a **vascular smooth muscle** cell or pericyte.
DETD . . . trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine which is capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell proliferation. Functional equivalents and derivatives of the aforementioned chemical compound are also included within the scope of the term. . .
DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta.
DETD TGF-beta activators or production stimulators of the invention are useful for inhibiting the pathological proliferation of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .
DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.
DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 1. This mechanism is believed to constitute a portion of the mechanism that

maintains **vascular smooth muscle** cells in
a non-proliferative state in healthy vessels.

DETD **Vascular smooth muscle** cell proliferation
is inhibited by an active form of TGF-beta. Tamoxifen has been shown by
the experimentation detailed in Example. . . TGF-beta from inactive
complexes present in serum. TGF-beta neutralizing antibodies inhibit
the
activity of TGF-beta, thereby facilitating the proliferation of
vascular smooth muscle cells. The apparent
in vivo physiological regulator of the activation of TGF-beta is
plasmin. Plasmin is derived from plasminogen through. . . the
lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the
activation of the latent form of TGF-beta and facilitating
proliferation
of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular
smooth muscle** cell proliferation is shown in FIG. 2.
Resting smooth muscle cells constitute cells in their normal, quiescent
non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad.
Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic
vascular smooth muscle cells derived from
healthy transplant donor tissue, cultured in Dulbecco's modified Eagles
medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular
smooth muscle** cells stimulated their proliferation in
a dose dependent manner (addition of 500 nM Lp(a) to human
vascular smooth muscle cells caused a
reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in
order to become capable of inhibiting the proliferation of
vascular smooth muscle cells. Plasmin is
therefore a leading candidate to be a physiological regulator of
TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human
vascular smooth muscle cells by interfering
with activation of latent TGF-beta was tested. In support of this
hypothesis, an observation was made that plasmin activity associated
with **vascular smooth muscle** cells was
reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in
the conditioned medium was also reduced by Lp(a) and apo(a) by about
2-fold, but was much lower than cell-associated plasmin activity in
vascular smooth muscle cell cultures. These
observations are consistent with previous findings that Lp(a) is a more
potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of
plasminogen activators rather than plasminogen activation, plasminogen
activator levels in human **vascular smooth
muscle** cell cultures were measured in the presence and absence
of the lipoproteins and in the presence of a large excess. . . as
competitive inhibitors. Total plasminogen activator activity was not
affected by the presence of any of the lipoproteins in the
vascular smooth muscle cell cultures. For
example, plasminogen activator activity in the conditioned medium
remained at 0.7+/-0.6 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead
to the conclusion that Lp(a) stimulates proliferation of human
vascular smooth muscle cells by inhibiting
plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting
by
binding active TGF-beta as well as reducing plasmin activity, human
vascular smooth muscle cells were cultured
in the presence of Lp(a). These cells had a population doubling time of
47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in

which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 (a concentration resulting in 50% inhibition) at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 1 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 2 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis

and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that are. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued lower dose delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD Human **vascular smooth muscle** cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat (doubling time. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation

DETD Cell culture, DNA synthesis assay and cell counting. Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD RNA Preparation and Northern Analysis. Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD Results. **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of

tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis
following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614+/-1714. . . time course of entry into
DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol.,
109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.
When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,.
. .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-betal mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured
rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Materials. Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. **Tamoxifen** (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were purchased from Flow Laboratories. 6-[.sup.3. . . factor and insulin-like growth facter 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Antiotensin II and **endothelin** 1 were obtained from Sigma and dissolved in sterile MilliQ water.

TGF-beta (0.5 .mu.g, lyophilized solid) was purchased from Peninsula,. . .

L7 ANSWER 4 OF 19 USPATFULL
AN 2000:84327 USPATFULL
TI Enhanced anti-angiogenic activity of permanently charged derivatives of steroid hormones
IN Biegon, Anat, Tel Aviv, Israel
Brewster, Marcus E., Gainesville, FL, United States
PA Pharmos Corporation, New York, NY, United States (U.S. corporation)
PI US 6083990 20000704
AI US 1997-833074 19970402 (8)
DT Utility
EXNAM Primary Examiner: Jones, Dwayne C.
LREP Pennie & Edmonds LLP
CLMN Number of Claims: 14
ECL Exemplary Claim: 1
DRWN 13 Drawing Figure(s); 6 Drawing Page(s)
LN.CNT 936
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
DETD . . . higher doses at more frequent intervals. It is further anticipated that the treatment of certain conditions known to involve abnormal **vascular smooth muscle** cell proliferation, including restenosis, will be treated beneficially with compositions according to the present invention.
DETD To assess potential mechanisms of action, the **tamoxifen** analogs, **tamoxifen** methiodide (TMI), **tamoxifen** benzyl bromide (TBB) and **tamoxifen** itself were examined in various biochemical assays related to angiogenesis. Blood vessel formation and destruction involves a series of events in which the basement membranes of existing vessels degrade, followed by **endothelial** cell migration, proliferation and re-establishment of the basement membrane. Thus, the effect of TMI on the ability of bovine **endothelial** cell to assemble into tubes and elongate when seeded in a basement membrane preparation (Matrigel) was evaluated.
Furthermore, since angiogenesis involves degradation of basement membrane proteins (including type IV collagen), the efficacy of TMI, TBB and **tamoxifen** in blocking matrix metalloprotease activity was examined through zymography using both whole cell (human fibrosarcoma and bovine **endothelial** cells) and cell-free systems. Finally, the specific action of TMI on transcription of various collagenases was investigated.
DETD In the case of **tamoxifen**, the speed with which necrosis occurs, and the finding that the number of capillaries in the tumor is reduced, has led to the proposition that **tamoxifen** exerts in vivo anti-angiogenic action resulting in tumor starvation and the observed cell death (Furman-Haran et al., Cancer Res. 54,. . . the percentage of various microscopic fields (250 .mu.m.sup.2 grid) containing capillaries (as defined by either red blood cells or distinct **endothelial** cells) was estimated (FIG. 3).
DETD FIG. 7 shows the effect of 20 .mu.M **tamoxifen** or TMI on collagenase activity for both the 72 kDa and 92 kDa isoform systems, as determined by zymography at. . . hours after drug administration. In addition, TMI proved to be a potent inhibitor of collagenase (gelatinase A) activity in bovine **endothelial** cells with an IC.sub.50 of between 1 and 5 .mu.M (FIG. 8).
DETD 4. Studies in transfected CAT systems in both fibrosarcoma and **endothelial** cells indicate that TMI inhibits matrix metalloproteases at the level of biosynthesis or expression. Furthermore, the lack of activity of the **tamoxifen** analog in cell free systems indicates that TMI does not directly inhibit the enzymes.

L7 ANSWER 5 OF 19 USPATFULL
AN 2000:73925 USPATFULL
TI Therapeutic inhibitor of **vascular smooth muscle** cells
IN Kunz, Lawrence L., Redmond, WA, United States
Klein, Richard A., Lynnwood, WA, United States
Reno, John M., Brier, WA, United States
Grainger, David J., Cambridge, United Kingdom
Metcalfe, James C., Cambridge, United Kingdom
Weissberg, Peter L., Cambridge, United Kingdom
Anderson, Peter G., Birmingham, AL, United States
PA NoeRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 6074659 20000613
AI US 1998-113733 19980710 (9)
RLI Continuation of Ser. No. US 1995-450793, filed on 25 May 1995, now patented, Pat. No. US 5811447 which is a continuation of Ser. No. US 1993-62451, filed on 13 May 1993, now abandoned which is a continuation-in-part of Ser. No. US 1993-11669, filed on 28 Jan 1993, now abandoned which is a continuation-in-part of Ser. No. WO 1992-US8220, filed on 25 Sep 1992 which is a continuation-in-part of Ser. No. US 1991-767254, filed on 27 Sep 1991, now abandoned
DT Utility
EXNAM Primary Examiner: Barts, Samuel
LREP Schwegman, Lundberg Woessner & Kluth P.A.
CLMN Number of Claims: 26
ECL Exemplary Claim: 1
DRWN 29 Drawing Figure(s); 21 Drawing Page(s)
LN.CNT 4818
TI Therapeutic inhibitor of **vascular smooth muscle** cells
AB . . . disease in a mammalian host, comprising administering to the host a therapeutically effective dosage of a therapeutic conjugate containing a **vascular smooth muscle** binding protein that associates in a specific manner with a cell surface of the **vascular smooth muscle** cell, coupled to a therapeutic agent dosage form that inhibits a cellular activity of the muscle cell. Methods are also provided for the direct and/or targeted delivery of therapeutic agents to **vascular smooth muscle** cells that cause a dilation and fixation of the vascular lumen by inhibiting smooth muscle cell contraction, thereby constituting a biological stent. Also discussed are mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation, agents that impact those mechanisms and protocols for the use of those agents.
SUMM . . . smooth muscle proteins is also described. The invention also relates to the direct or targeted delivery of therapeutic agents to **vascular smooth muscle** cells that results in dilation and fixation of the vascular lumen (biological stenting effect). Combined administration of a cytocidal conjugate and a sustained release dosage form of a **vascular smooth muscle** cell inhibitor is also disclosed. Mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation, agents that impact those mechanisms and protocols for the use of those agents are discussed.
SUMM . . . smooth muscle cell proliferation. It would be highly advantageous to develop new methods for inhibiting stenosis due to proliferation of **vascular smooth muscle** cells following traumatic injury to vessels such as occurs during vascular surgery. In addition, delivery of compounds that produce inhibitory effects of extended duration to the **vascular smooth muscle** cells would be advantageous. Local administration of such sustained release compounds would also be useful in the treatment of other. . .
SUMM In one aspect of the invention, new therapeutic methods and therapeutic

conjugates are provided for inhibiting **vascular smooth muscle** cells in a mammalian host. The therapeutic conjugates contain a **vascular smooth muscle** binding protein or peptide that binds in a specific manner to the cell membranes of a **vascular smooth muscle** cell or an interstitial matrix binding protein/peptide that binds in a specific manner to interstitial matrix (e.g., collagen) of the. . . after angioplasty or other vascular surgical procedures. The therapeutic conjugates of the invention achieve these advantageous effects by associating with **vascular smooth muscle** cells and pericytes, which may transform into smooth muscle cells. The therapeutic conjugate may contain: (1) therapeutic agents that alter.

. the like) are also contemplated for use in accordance with the present invention. Other aspects of the invention relate to **vascular smooth muscle** binding proteins that specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a **vascular smooth muscle** cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments the **vascular smooth muscle** binding protein binds to a CSPG target on the cell surface with an association constant of at least 10.sup.-4 M. In another preferred embodiment, the **vascular smooth muscle** binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal. . .

SUMM . . . therapeutic methods and therapeutic dosage forms involving sustained release of therapeutic agent to target cells. Preferably, the target cells are **vascular smooth muscle** cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that. . . the dosage form. Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting **vascular smooth muscle** cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, contraction, migration or the like) but does not kill the cell and, optionally, a **vascular smooth muscle** cell binding protein. Also, the methods and dosage forms of this aspect of the present invention are useful for inhibiting. .

SUMM . . . therapeutically significant target cell activity without killing the target cell, or target cell killing activity. For treatment of restenosis of **vascular smooth muscle** cells, useful therapeutic agents inhibit target cell activity (e.g., proliferation or migration) without killing the target cells. Preferred therapeutic moieties. . .

SUMM . . . to a relevant target cell population by a binding protein or peptide. Preferred binding proteins/peptides of the present invention are **vascular smooth muscle** cell binding protein, tumor cell binding protein and immune system effector cell binding protein. Preferred **vascular smooth muscle** cell binding proteins specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a **vascular smooth muscle** cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments, the **vascular smooth muscle** binding protein binds to a CSPG target on the cell surface with an association constant of at least 10.sup.-4 M. In other preferred embodiments, the **vascular smooth muscle** binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal. . . in this embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells.

Preferred binding peptides of this type are specifically associated with collagen, reticulum fibers or other intercellular matrix compounds. Preferred. . .

SUMM . . . involving administration of free (i.e., non-targeted or non-binding partner associated) therapeutic agent to target cells. Preferably, the target cells are **vascular smooth muscle** cells and the therapeutic agent is an inhibitor of **vascular smooth muscle** cell contraction, allowing the normal hydrostatic pressure to dilate the vascular lumen. Such contraction inhibition may be achieved by actin. . . which is preferably achievable and sustainable at a lower dose level than that necessary to inhibit protein synthesis. Consequently, the **vascular smooth muscle** cells synthesize protein required to repair minor cell trauma and secrete interstitial matrix, thereby facilitating the fixation of the vascular. . . post-procedural angiogram. cytochalasins (which inhibit the polymerization of G- to F-actin which, in turn, inhibits the migration and contraction of **vascular smooth muscle** cells) are the preferred therapeutic agents for use in this embodiment of the present invention. Free therapeutic agent protocols of. . . of stenosis after angioplasty or other vascular surgical procedures. Preferably, free therapeutic agent is administered directly or substantially directly to **vascular smooth muscle** tissue. Such administration is preferably effected by an infusion catheter, to achieve a 10.sup.-3 to 10.sup.-12 M concentration of said. . .

SUMM Another embodiment of the present invention incorporates administration of a cytocidal targeted conjugate to destroy proliferating **vascular smooth muscle** cells involved in vascular stenosis. The mitogenic agents released after this biological arteromyectomy are prevented from stimulating the remaining viable **vascular smooth muscle** cells to proliferate and restenose the vessel by administration of the anti-contraction (anti-migration) or anti-proliferative sustained release agents of the. . .

SUMM . . . Such dosage forms are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .

DRWD FIG. 1A is a photomicrograph of **vascular smooth muscle** cells of a 24-year-old male patient.

DRWD FIG. 1B is a photomicrograph of **vascular smooth muscle** cells in an artery of a 24-year-old male patient with **vascular smooth muscle** binding protein bound to the cell surface and membrane. The patient received the **vascular smooth muscle** binding protein by i.v. administration 4 days before the arterial tissue was prepared for histology.

DRWD FIG. 2 depicts a first scheme for chemical coupling of a therapeutic agent to a **vascular smooth muscle** binding protein.

DRWD FIG. 3 depicts a second scheme for chemical coupling of a therapeutic agent to a **vascular smooth muscle** binding protein.

DRWD FIG. 4A graphically depicts experimental data showing rapid binding of **vascular smooth muscle** binding protein to marker-positive test cells in vitro.

DRWD FIG. 4B graphically depicts experimental data showing rapid binding of **vascular smooth muscle** binding protein to **vascular smooth muscle** cells in vitro.

DRWD . . . data showing undesirable cytotoxicity of even low levels of therapeutic conjugate (i.e., RA-NR-AN-01), and the free RA therapeutic

agent, when **vascular smooth muscle** cells were treated for 24 hours in vitro.

DRWD . . . RA therapeutic agent was non-specifically cytotoxic, the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on cellular activity in **vascular smooth muscle** cells, as evidenced by metabolic activity in BO54 cells that were allowed a 48 hour recovery period prior to testing.

DRWD FIG. 10A graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of suramin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10B graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of staurosporin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10C graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of nitroglycerin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10D graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of cytochalasin B with respect to **vascular smooth muscle** cells.

DRWD FIGS. 15 and 16 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.

DETD "Therapeutic conjugate" means a **vascular smooth muscle** or an interstitial matrix binding protein coupled (e.g., optionally through a linker) to a therapeutic agent.

DETD . . . the conjugate aspects of the present invention to mean a molecule recognized in a specific manner by the matrix or **vascular smooth muscle** binding protein, e.g., an antigen, polypeptide antigen or cell surface carbohydrate (e.g., a glycolipid, glycoprotein, or proteoglycan) that is expressed on the cell surface membranes of a **vascular smooth muscle** cell or a matrix structure.

DETD . . . covalent or non-covalent chemical association (i.e., hydrophobic as through van der Waals forces or charge-charge interactions) of the matrix or **vascular smooth muscle** binding protein with the therapeutic agent. Due to the nature of the therapeutic agents employed, the binding proteins will normally. . .

DETD . . . transcription and translation with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., CSPG synthesized by a **vascular smooth muscle** cell or pericyte.

DETD "Cytochalasin" includes fungal metabolites exhibiting an inhibitory effect on target cellular metabolism, including prevention of contraction or migration of **vascular smooth muscle** cells. Preferably, cytochalasins inhibit the polymerization of monomeric actin (G-actin) to polymeric form (F-actin), thereby inhibiting cell functions requiring cytoplasmic. . .

DETD . . . trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethyl-ethylamine which is capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell proliferation.

Evidence exists that tamoxifen also acts to stabilize or organize areas of smooth muscle cell trauma. This organization/stabilization. . .

DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved. Functional equivalents of TGF-beta are, for example, moieties capable of. . .

DETD . . . negative immuno-staining with antibodies to cytokeratins (i.e., epithelial and fibroblast markers) and von Willdebrand factor (i.e., an

endothelial marker). Both **vascular smooth muscle** cells and pericytes are positive by immunostaining with the NR-AN-01 monoclonal antibody.

DETD The therapeutic conjugates and dosage forms of the invention are useful for inhibiting the activity of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .

DETD Therapeutic conjugates of the invention are obtained by coupling a **vascular smooth muscle** binding protein to a therapeutic agent. In the therapeutic conjugate, the **vascular smooth muscle** binding protein performs the function of targeting the therapeutic conjugate to **vascular smooth muscle** cells or pericytes, and the therapeutic agent performs the function of inhibiting the cellular activity of the smooth muscle cell. . .

DETD Nanoparticulate sustained release therapeutic dosage forms of preferred embodiments of the present invention are biodegradable and bind to the **vascular smooth muscle** cells and enter such cells primarily by endocytosis. The biodegradation of such nanoparticulates occurs over time (e.g., 10 to 21. . .

DETD Useful **vascular smooth muscle** binding protein is a polypeptide, peptidic, or mimetic compound (as described below) that is capable of binding to a target or marker on a surface component of an intact or disrupted **vascular smooth muscle** cell in such a manner that allows for either release of therapeutic agent extracellularly in the immediate interstitial matrix with. . . into an intracellular compartment of the entire targeted biodegradable moiety, permitting delivery of the therapeutic agent. Representative examples of useful **vascular smooth muscle** binding proteins include antibodies (e.g., monoclonal and polyclonal affinity-purified antibodies, F(ab').sub.2, Fab', Fab, and Fv fragments and/or complementarity determining regions. . .

DETD . . . dosage form embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the **vascular smooth muscle** cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and. . .

DETD Therapeutic agents of the invention are selected to inhibit a cellular activity of a **vascular smooth muscle** cell, e.g., proliferation, migration, increase in cell volume, increase in extracellular matrix synthesis (e.g., collagens, proteoglycans, and the like), or. . . spindle fiber formation (e.g., a drug such as colchicine) and the like; or b) as an inhibitor of migration of **vascular smooth muscle** cells from the medial wall into the intima, e.g., an "anti-migratory agent" such as a cytochalasin; or c) as an. . .

DETD . . . et al., U.S. Pat. No. 4,897,255), protein kinase inhibitors (e.g., staurosporin), stimulators of the production or activation of TGF-beta, including **tamoxifen** and functional equivalents or derivatives thereof, TGF-beta or functional equivalents, derivatives or analogs thereof, taxol or analogs thereof (e.g., taxotere),. . . e.g., cytokines (e.g., interleukins such as IL-1), growth factors, (e.g., PDGF, TGF-alpha or -beta, tumor necrosis factor, smooth muscle- and **endothelial**-derived growth factors, i.e., **endothelin**, FGF), homing receptors (e.g., for platelets or leukocytes), and extracellular matrix receptors (e.g., integrins). Representative examples of useful therapeutic agents. . .

DETD . . . as well as diminish smooth muscle cell proliferation following

angioplasty. The organization or stabilization may stem from the blockage of **vascular smooth muscle** cell maturation in to a pathologically proliferating form.

DETD For the sustained release dosage form embodiments of the present invention, therapeutic agents preferably are those that inhibit **vascular smooth muscle** cell activity without killing the cells (i.e., cytostatic therapeutic agents). Another way to define a cytostatic agent is a moiety. . . or more of the following capabilities: to inhibit DNA synthesis prior to protein synthesis inhibition or to inhibit migration of **vascular smooth muscle** cells into the intima. These therapeutic agents do not significantly inhibit protein synthesis (i.e., do not kill the target cells). . .

DETD **Vascular smooth muscle** binding proteins of the invention bind to targets on the surface of **vascular smooth muscle** cells. It will be recognized that specific targets, e.g., polypeptides or carbohydrates, proteoglycans and the like, that are associated with the cell membranes of **vascular smooth muscle** cells are useful for selecting (e.g., by cloning) or constructing (e.g., by genetic engineering or chemical synthesis) appropriately specific **vascular smooth muscle** binding proteins. Particularly useful "targets" are internalized by smooth muscle cells, e.g., as membrane constituent antigen turnover occurs in renewal.. . . endocytosis and the like. In a preferred embodiment, such a "target" is exemplified by chondroitin sulfate proteoglycans (CSPGs) synthesized by **vascular smooth muscle** cells and pericytes, and a discrete portion (termed an epitope herein) of the CSPG molecule having an apparent molecular weight. . . is a component of a larger 400 kD proteoglycan complex (14). In one presently preferred embodiment of the invention, a **vascular smooth muscle** binding protein is provided by NR-AN-01 monoclonal antibody (a subculture of NR-ML-05) that binds to an epitope in a **vascular smooth muscle** CSPG target molecule. The monoclonal antibody designated NR-ML-05 reportedly binds a 250 kD CSPG synthesized by melanoma cells (Morgan et. . . and functionally equivalent to, subclone NR-AN-01, disclosed herein. It will be recognized that NR-AN-01 is just one example of a **vascular smooth muscle** binding protein that specifically associates with the 400 kD CSPG target, and that other binding proteins associating with this target. . . human chimeric monoclonal antibodies have also been selected, as described herein, that specifically target to the 250 kD CSPG of **vascular smooth muscle** cells. The antibodies also appear to be internalized by the smooth muscle cells following binding to the cell membrane. Immunoreactivity. . . No. 4,879,225). In this disclosure and other human clinical studies, HAbs directed to the CSPG 250 kD antigen localized to **vascular smooth muscle** cells in vivo. Further, it will be recognized that the amino acid residues involved in the multi-point kinetic association of. . . molecular model for constructing functional equivalents, e.g., short polypeptides ("minimal polypeptides"), that have binding affinity for a CSPG synthesized by **vascular smooth muscle** cells and pericytes.

DETD . . . antibodies or fragments, for use in the practice of the invention have a binding affinity of >10.sup.4 liter/mole for the **vascular smooth muscle** 250 kD CSPG, and also the ability to be bound to and internalized by smooth muscle cells or pericytes.

DETD . . . to achieve the proper spacing for binding to the amino acids of, for example, an NR-AN-01 CSPG target synthesized by **vascular smooth muscle** cells or pericytes.

DETD . . . will be recognized that the inventors also contemplate the utility of human monoclonal antibodies or "humanized" murine antibody as

a **vascular smooth muscle** binding protein in the therapeutic conjugates of their invention. For example, murine monoclonal antibody may be "chimerized" by genetically recombining. . . residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics. Humanized **vascular smooth muscle** binding partners will be recognized to have the advantage of decreasing the immunoreactivity of the antibody or polypeptide in the. . .

DETD . . . release dosage forms of the present invention are those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the **vascular smooth muscle** cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and. . .

DETD Representative "coupling" methods for linking the therapeutic agent through covalent or non-covalent bonds to the **vascular smooth muscle** binding protein include chemical cross-linkers and heterobifunctional cross-linking compounds (i.e., "linkers") that react to form a bond between reactive groups. . . hydroxyl, amino, amido, or sulfhydryl groups) in a therapeutic agent and other reactive groups (of a similar nature) in the **vascular smooth muscle** binding protein. This bond may be, for example, a peptide bond, disulfide bond, thioester bond, amide bond, thioether bond, and. . . reference, is instructive of coupling methods that may be useful. In one presently preferred embodiment, the therapeutic conjugate contains a **vascular smooth muscle** binding protein coupled covalently to a trichothecene drug. In this case, the covalent bond of the linkage may be formed between one or more amino, sulfhydryl, or carboxyl groups of the **vascular smooth muscle** binding protein and a) the trichothecene itself; b) a trichothecene hemisuccinate carboxylic acid; c) a trichothecene hemisuccinate (HS) N-hydroxy succinimidate. . .

DETD The choice of coupling method will be influenced by the choice of **vascular smooth muscle** binding protein or peptide, interstitial matrix binding protein or peptide and therapeutic agent, and also by such physical properties as,. . .

DETD . . . result in increased smooth muscle in the intimal region of a traumatized vascular site, e.g., following angioplasty, e.g., pericytes and **vascular smooth muscle** cells. Aspects of the invention relate to therapeutic modalities in which the therapeutic conjugate of the invention is used to. . .

DETD . . . example, this therapeutically effective dosage is achieved by preparing 10 ml of a 200 .mu.g/ml therapeutic conjugate solution, wherein the **vascular smooth muscle** protein binding protein is NR-AN-01 and the therapeutic agent is Roridin A, a trichothecene drug. For treating vascular trauma, e.g.,. . . therapeutic conjugate according to the invention will be dependent on several factors, including, e.g.: a) the binding affinity of the **vascular smooth muscle** binding protein in the therapeutic conjugate; b) the atmospheric pressure applied during infusion; c) the time over which the therapeutic. . .

DETD . . . extracellularly is distributed within the relevant intracellular compartment; and (3) the therapeutic agent inhibits the desired cellular activity of the **vascular smooth muscle** cell, e.g., proliferation, migration, increased cellular volume, matrix synthesis, cell contraction and the like described above.

DETD Advantageously, non-coupled **vascular smooth muscle** cell binding protein (e.g., free NR-AN-01 antibody) is

administered prior to administration of the therapeutic agent conjugate or dosage form to provide a blocker of non-specific binding to cross-reactive sites. Blocking of such sites is important because **vascular smooth muscle** cell binding proteins will generally have some low level of cross-reactivity with cells in tissues other than the desired smooth. . . the specific vascular site, e.g., by making more of the therapeutic conjugate available to the cells. As an example, non-coupled **vascular smooth muscle** binding protein is administered from about 5 minutes to about 48 hours, most preferably from about 5 minutes to about. . . of minimizing displacement of the therapeutic conjugate or dosage form while maximizing blocking of the non-specific cross-reactive sites. The non-coupled **vascular smooth muscle** cell binding protein is administered in an amount effective to blocking binding of a least a portion of the non-specific. . .

DETD In addition, a second irrelevant **vascular smooth muscle** cell binding protein may optionally be administered to a patient prior to administration of the therapeutic conjugate or dosage form. . .

DETD . . . therapeutic agent. The cytocidal conjugate includes a binding partner (such as a protein or peptide) capable of specifically localizing to **vascular smooth muscle** cells and an active agent capable of killing such cells. The cytocidal conjugate is administered, preferably intravenously or through any. . . events. This cellular destruction causes the release of mitogens and other metabolic events, which events generally lead, in turn, to **vascular smooth muscle** cell proliferation. The sustained release anti-proliferative or anti-contractile dosage forms of the present invention are next administered, preferably through an infusion catheter or any convenient dosage form therefor. The sustained release dosage form retards the **vascular smooth muscle** cell proliferation and/or migration and contraction, thereby maintaining luminal diameter. This treatment methodology constitutes a biological arteromyectomy useful in stenotic vessels resulting from **vascular smooth muscle** cell hyperplasia and the like.

DETD . . . hours (preferably 24 to 72), an effective amount of a, for example, Pseudomonas exotoxin-monoclonal antibody conjugate capable of localizing to **vascular smooth muscle** cells is locally administered (e.g., via a catheter during an angioplasty procedure); and

DETD . . . embodiment of this aspect of the present invention involves administration of a therapeutic agent capable of inhibiting the ability of **vascular smooth muscle** cells to contract. Exemplary agents useful in the practice of this aspect of the present invention are those capable of. . . occur directly or indirectly through, for example, inhibition of calcium modulation, phosphorylation or other metabolic pathways implicated in contraction of **vascular smooth muscle** cells.

DETD Cytochalasins are exemplary therapeutic agents capable of generating a biological stenting effect on **vascular smooth muscle** cells. Cytochalasins are thought to inhibit both migration and contraction of **vascular smooth muscle** cells by interacting with actin. The cytochalasins interact with the ends of filamentous actin to inhibit the elongation of the. . . filaments. Low doses of cytochalasins (e.g., cytochalasin B) also disrupt microfilament networks of actin. In vitro data indicate that after **vascular smooth muscle** cells clear cytochalasin B, the cells regenerate enough polymerized actin to resume migration within about 24 hours. In vivo assessments reveal that

vascular smooth muscle cells regain vascular tone within 2 to 4 days. It is during this recuperative period that the lumen diameter fixation. . .

DETD Inhibition of **vascular smooth muscle** cell migration (from the tunica media to the intima) has been demonstrated in the same dose range (Example 11); however,. . . sustained exposure of the vessel to the therapeutic agent is preferable in order to maximize these anti-migratory effects. If the **vascular smooth muscle** cells cannot migrate into the intima, they cannot proliferate there. Should **vascular smooth muscle** cells migrate to the intima, a subsequently administered anti-proliferative sustained release dosage form inhibits the intimal proliferation. As a result,. . .

DETD (ii) inhibits target cell proliferation (e.g, following 5 minute and 24 hour exposure to the agent, in vitro **vascular smooth muscle** tissue cultures demonstrate a level of inhibition of .sup.3 H-thymidine uptake and, preferably, display relatively less inhibition of .sup.3 H-leucine. . .

DETD . . . or more of the preceding attributes, the agent is subjected to a second testing protocol that involves longer exposure of **vascular smooth muscle** cells to the therapeutic agent.

DETD (i) upon long term (e .g, 5 days) exposure, the agent produces the same or similar in vitro effect on **vascular smooth muscle** tissue culture DNA synthesis and protein synthesis, as described above for the 5 minute and 24 hour exposures; and

DETD . . . pig femoral artery model. Preferably, such agents demonstrate a 50% or greater inhibition of cell proliferation in the tunica media **vascular smooth muscle** cells, as indicated by a 1 hour "BRDU flash labeling" prior to tissue collection and histological evaluation. If an agent. . . to permit intravenous administration to achieve the 50% inhibition, or if the agent is amenable to local delivery to the **vascular smooth muscle** cells with sustained release at an effective anti-proliferative dose. Sustained release agents are evaluated in a sustained release dosage form. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 15. This mechanism is believed to constitute a portion of the mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels. The pathway has been elucidated by the inventors of a patent application. . .

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. The apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 16.

Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEN)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7+/-0.06 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 16). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell

proliferation is set forth in detail in Example 16. The results of those
experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 15 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 16 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta, TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued lower dose delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD . . . involve the administration of taxol or analogs thereof in soluble or sustained release dosage form. Taxol is believed to stabilize

vascular smooth muscle cells against
division by binding to microtubules and inhibiting the organization and ordering of the microtubule network. Cell migration may. . .

DETD Binding to **Vascular Smooth Muscle** Cells In the Blood Vessel Wall In Vivo

DETD . . . (FIG. 1A and FIG. 1B). This photomicrograph (FIG. 1B) demonstrates the ability of the MAb to specifically bind to human **vascular smooth muscle** in vivo, and to be internalized by the cells and remain in the cells for extended periods.

DETD . . . conducted to determine the binding kinetics of a smooth muscle binding protein with a Ka of >10.sup.9 liter/mole. Because human **vascular smooth muscle** cells grow slowly in culture, and baboon smooth muscle cells were found to express the human CSPG cell surface marker,. . .

DETD . . . to determine, in a domestic pig model system, the infusion conditions suitable for delivery of a therapeutic conjugate to the **vascular smooth muscle** cells in carotid and femoral arteries. Delivery conditions were monitored by evaluating the penetration of the therapeutic conjugate into the vascular wall, and specific binding of the therapeutic conjugate to the **vascular smooth muscle** cells in the vessel wall.

DETD . . . wall of swine coronary and femoral arteries 3-5 days after surgery, and the NR-AN-01 appeared to be associated only with **vascular smooth muscle** cells. These findings suggest that NR-AN-01 is capable of specifically binding to its target antigen in vivo.

DETD Inhibition of **Vascular Smooth Muscle** Cells In Vivo

DETD . . . response to vascular trauma, including restenosis following angioplasty. Domestic pigs were used to study the effects of NR-AN-01 (i.e., termed **vascular smooth muscle** binding protein or simply VSMBP in these studies; and therapeutic conjugates with Roridin A are termed VSMBP - RA). The. . .

DETD . . . human coronary arteries. The test protocol was designed as an initial in vivo screening of intra-arteriolar, site specific, catheter administered, **vascular smooth muscle** binding protein (VSMBP) conjugates. Toxicity of free drug was also evaluated, i.e., for pathobiological effects on arteriolar smooth muscle cells.. . .

DETD . . . with H & E, Massons Trichrome and Movats Pentachrome for morphological studies. Sections were also used for immunohistological staining of **vascular smooth muscle**.

DETD **Vascular Smooth Muscle** Cell In Vitro DNA and Protein Synthesis Inhibition

DETD The ability of various therapeutic agents to inhibit DNA synthesis and protein synthesis in **vascular smooth muscle** cells was tested. .sup.3 H-leucine and .sup.3 H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

DETD 5 minute exposure: .sup.3 H-leucine uptake: **Vascular smooth muscle** cells at 40,000 cells/ml were seeded in sterile 24 well plates at 1 ml/well. The plates were incubated overnight at. . . CO.sub.2, 95% air in a humidified atmosphere (saturation). Log dilutions of the therapeutic agent of interest were incubated with the **vascular smooth muscle** cells for 5 minutes or 24 hours. Samples of the therapeutic agents were diluted in DMEM:F-12 medium (Whittaker Bioproducts, Walkersville,. . .

DETD 5 minute exposure; .sup.3 H-thymidine uptake: **Vascular smooth muscle** cells were incubated in complete medium with 5% FBS (Gibco) overnight at 37.degree. C. in a humidified, 5% CO.sub.2 environment. . .

DETD **Vascular smooth muscle** cells were seeded at 4.0.times.10.sup.4 cells/ml medium/well on a commercially prepared four well slide (Nunc, Inc., Naperville, Ill.). Enough slides. . .

DETD . . . the practice of sustained release dosage form embodiments of the present invention. More specifically, the compounds inhibited the ability of **vascular smooth muscle** cells to undergo DNA synthesis in the presence of 5% FBS to a greater extent than they inhibited protein synthesis of **vascular smooth muscle** cells. The protein and DNA synthesis inhibitory effects of suramin, staurosporin, nitroglycerin and cytochalasin B during a 5 minute and. . .

DETD Specific Binding and Internalization of Targeted Particles by **Vascular Smooth Muscle** Cells

DETD The ability of **vascular smooth muscle** cells to bind and internalize particles coated with binding protein or peptide was demonstrated with monoclonal antibody (NR-AN-01) coated gold beads both in vitro and in vivo. The **vascular smooth muscle** cell tissue cultures (B054), an antigen positive control cell line (A375) and an antigen negative control cell line (HT29) were. . .

DETD . . . gold beads devoid of NR-AN-01 to surface mucin produced by HT29 cells was observed, resulting in modest non-specific internalization thereof. **Vascular smooth muscle** cell uptake of NR-AN-01 targeted gold beads was highly specific in cell suspension cultures.

DETD The targeted gold bead **vascular smooth muscle** cell surface binding, internalization and lysosome concentration was observed in vivo as well. NR-AN-01 coated gold beads were infused via. . . pig femoral artery immediately following balloon trauma. The bead internalization rate varied with the degree of damage sustained by the **vascular smooth muscle** cell during the balloon trauma. Cells with minimal or no damage rapidly internalized the particles by endocytosis and phagocytosis, concentrating. . .

DETD **Vascular Smooth Muscle** In Vitro DNA and Protein Synthesis Inhibition By Staurosporin and Cytochalasin

DETD The ability of staurosporin and cytochalasin to inhibit in vitro DNA and protein synthesis in **vascular smooth muscle** cells was tested. .sup.3 H-leucine and .sup.3 H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

DETD **Vascular smooth muscle** cells at 40,000-50,000 cells/ml were seeded and processed as described in Example 8, "15 minute exposure; .sup.3 H-leucine uptake." Log. . . ml/well of each therapeutic agent dilution was added in quadruplicate wells, and the agent of interest was incubated with the **vascular smooth muscle** cells for 5 min at room temperature in a sterile ventilated hood. Following therapeutic agent incubation, the wells were subsequently. . .

DETD 5 Minute Exposure: DNA Synthesis Assay: **Vascular smooth muscle** (BO54) cells were seeded and processed in 24 well plates, as described above under "5 Minute Exposure: Protein Synthesis Assay.". . .

DETD **Vascular smooth muscle** (B054) cells at 20,000 cells/ml were seeded in sterile 24 well plates and incubated in complete medium (1 ml/well) overnight. . .

DETD **Vascular Smooth Muscle** Cell Migration Inhibition

DETD **Vascular smooth muscle** cells (B054) were derived from explants of baboon aortic smooth muscle, as described in Example 10. The cells were grown in flat bottom, six well tissue culture plates, which hold about 5 ml of medium. The **vascular**

smooth muscle cells were plated at 200,000 cells/well and placed at 37.degree. C. in a humidified 5% CO.sub.2 incubator for 18 hours.. . .

DETD . . . Migration Grade, wherein no migration; +1=minimal; +2=mild; +3=moderate; and +4 =marked (maximum density; limit of cell contact inhibition) migration of **vascular smooth muscle** cells into the cleared area adjacent to the scratch. In this Table, "T" denotes a morphological Toxicity Grade, wherein--=no toxicity;. . .

DETD The data indicate that cytochalasin B inhibits the migration (+1 to +2) of **vascular smooth muscle** cells into the cleared area adjacent to the scratch at a dose of 0.1 .mu.g/ml with only minimal (- to. . .

DETD TABLE 4

SCRATCH-MIGRATION ASSAY:
INHIBITION OF **VASCULAR SMOOTH MUSCLE** CELL MIGRATION BY CYTOCHALASIN B

	7-day Continuous Exposure Dosage .mu.g/mL				7-day Recovery Post Exposure Dosage .mu.g/mL	
	Control				Control	
Day	0.0	0.01	0.1	1.0	0.0.	. .

DETD Therapeutic Agent Cytotoxic Effects on **Vascular Smooth Muscle** Cells--Pulse and Continuous Exposure

DETD **Vascular smooth muscle** cells were exposed to a therapeutic agent in one of two exposure formats:

DETD In Vivo BRDU Assay: Inhibition of **Vascular Smooth Muscle** Cell Proliferation

DETD BRDU assay: In vivo **vascular smooth muscle** proliferation was quantitated by measuring incorporation of the base analog 5-bromo-2'-deoxyuridine (BRDU, available from Sigma Chemical Co.) into DNA during. . .

DETD . . . uptake relative to a PBS control; however, cytochalasin B and staurosporin inhibited BRDU uptake (i.e., cell proliferation) without killing the **vascular smooth muscle** cells.

The number of **vascular smooth muscle** cells labeled with BRDU was assigned a grade at 400X magnification as follows:

DETD . . . from 0.1 .mu.g/ml (.apprxeq.140% increase) to 1.0 .mu.g/ml (FIG. 14). The 10 .mu.g/ml dose appeared to be toxic to the **vascular smooth muscle** cells (data not shown). The subthreshold dose (0.01 .mu.g/ml) and negative control (PBS) exhibited a .+-..apprxeq.20% change in luminal area.. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation

DETD Cell culture, DNA synthesis assay and cell counting. Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991.

Vascular smooth muscle cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely

abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD RNA Preparation and Northern Analysis. Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD Results. **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614+/-1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth**

muscle cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-beta1 mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little MRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

CLM What is claimed is:

14. The method of claim 1 wherein the amount is effective to inhibit migration of **vascular smooth muscle** cells.

16. The method of claim 1 wherein the amount is effective to inhibit proliferation of **vascular smooth muscle** cells.

L7 ANSWER 6 OF 19 CAPLUS COPYRIGHT 2001 ACS

AN 2000:392207 CAPLUS

DN 133:117961

TI Endothelium modulates anion channel-dependent aortic contractions to iodide

AU Lamb, Fred S.; Barna, Thomas J.

CS Department of Pediatrics, University of Iowa, Iowa City, IA, 52242, USA

SO Am. J. Physiol. (2000), 278(5, Pt. 2), H1527-H1536
CODEN: AJPHAP; ISSN: 0002-9513

PB American Physiological Society

DT Journal

LA English

RE.CNT 43

RE

(1) Bolotina, V; Nature 1994, V368, P850 CAPLUS

(2) Brosius, F; Am J Physiol Renal Fluid Electolyte Physiol 1997, V273, PF1039 CAPLUS

(3) Byrne, N; J Physiol 1988, V404, P557 CAPLUS

(4) Carr, P; Am J Physiol Cell Physiol 1995, V268, PC580 CAPLUS

(6) Clemo, H; J Gen Physiol 1992, V100, P89 CAPLUS

ALL CITATIONS AVAILABLE IN THE RE FORMAT

AB Anion currents contribute to **vascular smooth muscle** (VSM) membrane potential. The substitution of extracellular chloride (Cl) with iodide (I) or bromide (Br) initially inhibited and then potentiated isometric contractile responses of rat aortic rings to norepinephrine. Anion substitution alone produced a small relaxation, which occurred despite a lack of active tone and minimal subsequent contraction of **endothelium**-intact rings (4.2 .+-. 1.2% of the response to 90 mM KCl). **Endothelium**-denuded rings underwent a similar initial relaxation but then contracted vigorously (I > Br). Responses to 130 mM I (93.7 .+-. 1.9% of 90 mM KCl) were inhibited by nifedipine (10-6 M), niflumic acid (10-5 M), **tamoxifen** (10-5 M), DIDS (10-4 M), and HCO3--free buffer (HEPES 10 mM) but not by

bumetanide (10-5 M). Intact rings treated with N.omega.-nitro-L-arginine (10-4 M) responded weakly to I (15.5 .+-. 2.1% of 90 mM KCl), whereas Hb (10-5 M), indomethacin (10-6 M), 17-octadecynoic acid (10-5 M), and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (10-6 M) all failed to augment the response of intact rings to I. We hypothesize that VSM takes up I primarily via an anion exchanger. Subsequent I efflux through anion channels having a selectivity of I > Br > Cl produces depolarization. In **endothelium**-denuded or agonist-stimulated vessels, this current is sufficient to activate voltage-dependent calcium channels and cause contraction. Neither nitric oxide nor prostaglandins are the primary **endothelial** modulator of these anion channels. If they are regulated by an **endothelium**-dependent hyperpolarizing factor it is not a cytochrome P 450 metabolite.

ST iodide bromide anion channel vasoconstriction **vascular smooth muscle**; chloride calcium channel anion exchanger membrane depolarization iodide vasoconstriction; nitric oxide vascular endothelium iodide vasoconstriction

L7 ANSWER 7 OF 19 CAPLUS COPYRIGHT 2001 ACS
AN 2000:568819 CAPLUS
DN 133:233023
TI 17.beta.-Estradiol increases intracellular calcium concentration through a short-term and nongenomic mechanism in rat vascular endothelium in culture
AU Rubio-Gayosso, Ivan; Sierra-Ramirez, Alfredo; Garcia-Vazquez, Alicia; Martinez-Martinez, Aline; Munoz-Garcia, Olga; Morato, Tomas; Ceballos-Reyes, Guillermo
CS Laboratorio Multidisciplinario, Seccion de Posgrado, Escuela Superior de Medicina, Instituto Politecnico Nacional de Mexico D.F., Mexico, 11340, Mex.
SO J. Cardiovasc. Pharmacol. (2000), 36(2), 196-202
CODEN: JCPCDT; ISSN: 0160-2446
PB Lippincott Williams & Wilkins
DT Journal
LA English
RE.CNT 34
RE
(1) Batra, S; Br J Pharmacol 1987, V92, P389 CAPLUS
(2) Baulieu, E; Nature 1978, V275, P593 CAPLUS
(3) Bayard, F; Endocrinology 1995, V136, P1523 CAPLUS
(4) Blackmore, P; J Biol Chem 1990, V265, P1376 CAPLUS
(5) Carson-Jurica, M; Endocr Rev 1990, V11, P201 CAPLUS
ALL CITATIONS AVAILABLE IN THE RE FORMAT

AB 17.beta.-Estradiol (E2) plays an important role in Ca2+ fluxes in several cell types. It has been proposed that some of its effects are of nongenomic origin. E2 at **vascular smooth muscle** level can block calcium entry through L-type calcium channels, this mechanism cannot include vascular **endothelial** cells (VECs), in which increases in the intracellular calcium concn. ([Ca2+]i) are necessary to NO synthesis. The authors used male rat aorta ECs in culture loaded with fura-2 and a fluorescence imaging system to evaluate the short-term effects of E2 on [Ca2+]i kinetics. The authors explored the participation of the intracellular steroid receptor on the effects induced by E2, using **tamoxifen** (1 .mu.M) and ICI 182.780 (10 .mu.M). The authors' results showed that E2 (like bradykinin) induced an increase in [Ca2+]i. Such agonist-like effects showed a biphasic curve behavior. The 17.beta.-estradiol effects were not modified by the presence of the intracellular estradiol-receptor antagonist **tamoxifen**, but it is blocked in the presence of the ICI 182,780. The 17.beta.-estradiol effects were obtained even with restriction of steroid-free diffusion into cells (17.beta.-estradiol-bovine serum albumin). Phospholipase C.beta. activity is involved in these effects,

because U-73122, a PLC.beta. inhibitor, blocked E2 effects. All E2 effects were of rapid onset (milliseconds), exerted at the membrane level, and of rapid offset. The authors conclude that estradiol can influence the **endothelium** physiol. responses through effects of nongenomic origin.

L7 ANSWER 8 OF 19 CAPLUS COPYRIGHT 2001 ACS
AN 1999:750378 CAPLUS
DN 132:103016
TI Endothelium-independent relaxation of **vascular smooth muscle** by 17.beta.-estradiol
AU Gonzales, Rayna J.; Kanagy, Nancy L.
CS Department of Cell Biology and Physiology, University of New Mexico School of Medicine, Albuquerque, NY, 87131-5218, USA
SO J. Cardiovasc. Pharmacol. Ther. (1999), 4(4), 227-234
CODEN: JCPTFE; ISSN: 1074-2484
PB Churchill Livingstone
DT Journal
LA English
RE.CNT 31
RE
(1) Batra, S; J Physiol 1978, V276, P329 CAPLUS
(2) Bond, M; J Physiol (London) 1984, V355, P677 CAPLUS
(3) Darkow, D; Am J Physiol 1997, V272, PH2765 CAPLUS
(4) Farhat, M; Biochem Pharmacol 1996, V51, P571 CAPLUS
(5) Farhat, M; J Pharmacol Exp Ther 1996, V276, P652 CAPLUS
ALL CITATIONS AVAILABLE IN THE RE FORMAT
TI Endothelium-independent relaxation of **vascular smooth muscle** by 17.beta.-estradiol
AB Estrogens directly dilate arteries, and this acute relaxation of **vascular smooth muscle** (VSM) may contribute to the cardioprotective effect of this important hormone. However, the mechanism by which estrogens relax VSM is not clear. Based on observations in isolated smooth muscle cells, the authors hypothesized that 17.beta.-estradiol (E2) causes dilation through receptor-mediated activation of K+ channels in VSM cells. To test this hypothesis, E2-relaxation was studied in arteries from male Sprague-Dawley rats. The authors obsd. that the estrogen receptor antagonist, **tamoxifen** (3 .mu.mol) attenuated E2 relaxation, suggesting that at least a portion of the relaxation depends on activation of E2 receptors. The nitric oxide synthase inhibitor, N.omega.-nitro-L-arginine (100 .mu.mol) did not affect E2 relaxation in either denuded or **endothelium**-intact arterial strips. Furthermore, inhibition of guanylyl cyclase with LY83583 (10 .mu.mol) had no effect on the relaxation, suggesting that nitric oxide does not contribute to this relaxation. Vascular segments contracted with 90 mmol KCl to disrupt the K+ gradient had a similar E2 relaxation to segments contracted with phenylephrine (10-6 M) indicating that E2 relaxation does not require K+-channel activation. Finally, E2 pretreatment inhibited contraction of arterial segments depleted of intracellular calcium but in the presence of extracellular calcium. However, E2 did not affect contraction of strips in calcium-free soln. These final expts. suggest that E2 inhibits Ca2+ influx but not intracellular calcium release. Together, these studies establish that E2 causes receptor-mediated relaxation of peripheral resistance arteries through inhibition of calcium entry independent of nitric oxide prodn., guanylyl cyclase stimulation, and K+-channel activation.
ST estradiol receptor calcium vasodilation **vascular smooth muscle**

L7 ANSWER 9 OF 19 USPATFULL
AN 1998:115762 USPATFULL

TI Therapeutic inhibitor of **vascular smooth muscle** cells
IN Kunz, Lawrence L., Redmond, WA, United States
Klein, Richard A., Lynnwood, WA, United States
Reno, John M., Brier, WA, United States
Grainger, David J., Cambridge, England
Metcalfe, James C., Cambridge, England
Weissberg, Peter L., Cambridge, England
Anderson, Peter G., Birmingham, AL, United States
PA NeoRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 5811447 19980922
AI US 4507932 19950525 (8)
RLI Continuation of Ser. No. 62451, filed on 13 May 1993, now abandoned which is a continuation-in-part of Ser. No. 11669, filed on 28 Jan 1993, now abandoned
DT Utility
EXNAM Primary Examiner: Barts, Samuel
LREP Schwegman, Lundberg, Woessner & Kluth, P.A.
CLMN Number of Claims: 18
ECL Exemplary Claim: 1
DRWN 29 Drawing Figure(s); 21 Drawing Page(s)
LN.CNT 4812
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
TI Therapeutic inhibitor of **vascular smooth muscle** cells
AB . . . disease in a mammalian host, comprising administering to the host a therapeutically effective dosage of a therapeutic conjugate containing a **vascular smooth muscle** binding protein that associates in a specific manner with a cell surface of the **vascular smooth muscle** cell, coupled to a therapeutic agent dosage form that inhibits a cellular activity of the muscle cell. Methods are also provided for the direct and/or targeted delivery of therapeutic agents to **vascular smooth muscle** cells that cause a dilation and fixation of the vascular lumen by inhibiting smooth muscle cell contraction, thereby constituting a biological stent. Also discussed are mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation, agents that impact those mechanisms and protocols for the use of those agents.
SUMM . . . smooth muscle proteins is also described. The invention also relates to the direct or targeted delivery of therapeutic agents to **vascular smooth muscle** cells that results in dilation and fixation of the vascular lumen (biological stenting effect). Combined administration of a cytocidal conjugate and a sustained release dosage form of a **vascular smooth muscle** cell inhibitor is also disclosed. Mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation, agents that impact those mechanisms and protocols for the use of those agents are discussed.
SUMM . . . smooth muscle cell proliferation. It would be highly advantageous to develop new methods for inhibiting stenosis due to proliferation of **vascular smooth muscle** cells following traumatic injury to vessels such as occurs during vascular surgery. In addition, delivery of compounds that produce inhibitory effects of extended duration to the **vascular smooth muscle** cells would be advantageous. Local administration of such sustained release compounds would also be useful in the treatment of other. . .
SUMM In one aspect of the invention, new therapeutic methods and therapeutic conjugates are provided for inhibiting **vascular smooth muscle** cells in a mammalian host. The therapeutic conjugates contain a **vascular smooth muscle** binding protein or peptide that binds in a specific manner to the cell membranes of a **vascular smooth muscle** cell or an

interstitial matrix binding protein/peptide that binds in a specific manner to interstitial matrix (e.g., collagen) of the. . . after angioplasty or other vascular surgical procedures. The therapeutic conjugates of the invention achieve these advantageous effects by associating with **vascular smooth muscle** cells and pericytes, which may transform into smooth muscle cells. The therapeutic conjugate may contain: (1) therapeutic agents that alter.

.

. the like) are also contemplated for use in accordance with the present invention. Other aspects of the invention relate to **vascular smooth muscle** binding proteins that specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a **vascular smooth muscle** cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments the **vascular smooth muscle** binding protein binds to a CSPG target on the cell surface with an association constant of at least 10.sup.-4 M. In another preferred embodiment, the **vascular smooth muscle** binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal. . .

SUMM . . . therapeutic methods and therapeutic dosage forms involving sustained release of therapeutic agent to target cells. Preferably, the target cells are **vascular smooth muscle** cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that. . . the dosage form. Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting **vascular smooth muscle** cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, contraction, migration or the like) but does not kill the cell and, optionally, a **vascular smooth muscle** cell binding protein. Also, the methods and dosage forms of this aspect of the present invention are useful for inhibiting. .

.

SUMM . . . therapeutically significant target cell activity without killing the target cell, or target cell killing activity. For treatment of restenosis of **vascular smooth muscle** cells, useful therapeutic agents inhibit target cell activity (e.g., proliferation or migration) without killing the target cells. Preferred therapeutic moieties. . .

SUMM . . . to a relevant target cell population by a binding protein or peptide. Preferred binding proteins/peptides of the present invention are **vascular smooth muscle** cell binding protein, tumor cell binding protein and immune system effector cell binding protein. Preferred **vascular smooth muscle** cell binding proteins specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a **vascular smooth muscle** cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments, the **vascular smooth muscle** binding protein binds to a CSPG target on the cell surface with an association constant of at least 10.sup.-4 M. In other preferred embodiments, the **vascular smooth muscle** binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal. . . in this embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Preferred binding peptides of this type are specifically associated with collagen, reticulum fibers or other intercellular matrix compounds. Preferred. . .

SUMM . . . involving administration of free (i.e., non-targeted or non-binding partner associated) therapeutic agent to target cells.

Preferably, the target cells are **vascular smooth muscle** cells and the therapeutic agent is an inhibitor of **vascular smooth muscle** cell contraction, allowing the normal hydrostatic pressure to dilate the vascular lumen. Such contraction inhibition may be achieved by actin. . . which is preferably achievable and sustainable at a lower dose level than that necessary to inhibit protein synthesis. Consequently, the **vascular smooth muscle** cells synthesize protein required to repair minor cell trauma and secrete interstitial matrix, thereby facilitating the fixation of the vascular. . . post-procedural angiogram. Cytochalasins (which inhibit the polymerization of G- to F-actin which, in turn, inhibits the migration and contraction of **vascular smooth muscle** cells) are the preferred therapeutic agents for use in this embodiment of the present invention. Free therapeutic agent protocols of. . .

of

stenosis after angioplasty or other vascular surgical procedures. Preferably, free therapeutic agent is administered directly or substantially directly to **vascular smooth muscle** tissue. Such administration is preferably effected by an infusion catheter, to achieve a 10.sup.-3 M to 10.sup.-12 M concentration of. . .

SUMM Another embodiment of the present invention incorporates administration of a cytocidal targeted conjugate to destroy proliferating **vascular smooth muscle** cells involved in vascular stenosis. The mitogenic agents released after this biological arteromyectomy are prevented from stimulating the remaining viable **vascular smooth muscle** cells to proliferate and restenose the vessel by administration of the anti-contraction (anti-migration) or anti-proliferative sustained release agents of the. . .

SUMM . . . Such dosage forms are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .

DRWD FIG. 1A is a photomicrograph of **vascular smooth muscle** cells of a 24-year-old male patient.

DRWD FIG. 1B is a photomicrograph of **vascular smooth muscle** cells in an artery of a 24-year-old male patient with **vascular smooth muscle** binding protein bound to the cell surface and membrane. The patient received the **vascular smooth muscle** binding protein by i.v. administration 4 days before the arterial tissue was prepared for histology.

DRWD FIG. 2 depicts a first scheme for chemical coupling of a therapeutic agent to a **vascular smooth muscle** binding protein.

DRWD FIG. 3 depicts a second scheme for chemical coupling of a therapeutic agent to a **vascular smooth muscle** binding protein.

DRWD FIG. 4A graphically depicts experimental data showing rapid binding of **vascular smooth muscle** binding protein to marker-positive test cells in vitro.

DRWD FIG. 4B graphically depicts experimental data showing rapid binding of **vascular smooth muscle** binding protein to **vascular smooth muscle** cells in vitro.

DRWD . . . data showing undesirable cytotoxicity of even low levels of therapeutic conjugate (i.e., RA-NR-AN-01), and the free RA therapeutic agent, when **vascular smooth muscle** cells were treated for 24 hours in vitro.

DRWD . . . RA therapeutic agent was non-specifically cytotoxic, the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on cellular activity in **vascular smooth muscle** cells, as evidenced by metabolic activity in BO54 cells

that were allowed a 48 hour recovery period prior to testing.

DRWD FIG. 10A graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of suramin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10B graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of staurosporin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10C graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of nitroglycerin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10D graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of cytochalasin B with respect to **vascular smooth muscle** cells.

DRWD FIGS. 15 and 16 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.

DETD "Therapeutic conjugate" means a **vascular smooth muscle** or an interstitial matrix binding protein coupled (e.g., optionally through a linker) to a therapeutic agent.

DETD . . . the conjugate aspects of the present invention to mean a molecule recognized in a specific manner by the matrix or **vascular smooth muscle** binding protein, e.g., an antigen, polypeptide antigen or cell surface carbohydrate (e.g., a glycolipid, glycoprotein, or proteoglycan) that is expressed on the cell surface membranes of a **vascular smooth muscle** cell or a matrix structure.

DETD . . . covalent or non-covalent chemical association (i.e., hydrophobic as through van der Waals forces or charge-charge interactions) of the matrix or **vascular smooth muscle** binding protein with the therapeutic agent. Due to the nature of the therapeutic agents employed, the binding proteins will normally. . .

DETD . . . transcription and translation with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., CSPG synthesized by a **vascular smooth muscle** cell or pericyte.

DETD "Cytochalasin" includes fungal metabolites exhibiting an inhibitory effect on target cellular metabolism, including prevention of contraction or migration of **vascular smooth muscle** cells. Preferably, cytochalasins inhibit the polymerization of monomeric actin (G-actin) to polymeric form (F-actin), thereby inhibiting cell functions requiring cytoplasmic. . .

DETD . . . trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethyl-ethylamine which is capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell proliferation. Evidence exists that tamoxifen also acts to stabilize or organize areas of smooth muscle cell trauma. This organization/stabilization. . .

DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved. Functional equivalents of TGF-beta are, for example, moieties capable of. . .

DETD . . . negative immuno-staining with antibodies to cytokeratins (i.e., epithelial and fibroblast markers) and von Willdebrand factor (i.e., an endothelial marker). Both **vascular smooth muscle** cells and pericytes are positive by immunostaining with the NR-AN-01 monoclonal antibody.

DETD The therapeutic conjugates and dosage forms of the invention are useful for inhibiting the activity of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating

stenosis following angioplasty. As used herein the term "reducing" means
decreasing the intimal. . .

DETD Therapeutic conjugates of the invention are obtained by coupling a **vascular smooth muscle** binding protein to a therapeutic agent. In the therapeutic conjugate, the **vascular smooth muscle** binding protein performs the function of targeting the therapeutic conjugate to **vascular smooth muscle** cells or pericytes, and the therapeutic agent performs the function of inhibiting the cellular activity of the smooth muscle cell. . .

DETD Nanoparticulate sustained release therapeutic dosage forms of preferred embodiments of the present invention are biodegradable and bind to the **vascular smooth muscle** cells and enter such cells primarily by endocytosis. The biodegradation of such nanoparticulates occurs over time (e.g., 10 to 21. . .

DETD Useful **vascular smooth muscle** binding protein is a polypeptide, peptidic, or mimetic compound (as described below) that is capable of binding to a target or marker on a surface component of an intact or disrupted **vascular smooth muscle** cell in such a manner that allows for either release of therapeutic agent extracellularly in the immediate interstitial matrix with. . . into an intracellular compartment of the entire targeted biodegradable moiety, permitting delivery of the therapeutic agent. Representative examples of useful **vascular smooth muscle** binding proteins include antibodies (e.g., monoclonal and polyclonal affinity-purified antibodies, F(ab').sub.2, Fab', Fab, and Fv
fragments and/or complementarity determining regions. . .

DETD . . . dosage form embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Such binding peptides deliver the therapeutic agent to the interstitial space
between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the **vascular smooth muscle** cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and. . .

DETD Therapeutic agents of the invention are selected to inhibit a cellular activity of a **vascular smooth muscle** cell, e.g., proliferation, migration, increase in cell volume, increase in extracellular matrix synthesis (e.g., collagens, proteoglycans, and the like), or. . . spindle fiber formation (e.g., a drug such as colchicine) and the like; or b) as an inhibitor of migration of **vascular smooth muscle** cells from the medial wall into the intima, e.g., an "anti-migratory agent" such as a cytochalasin; or c) as an. . .

DETD . . . et al., U.S. Pat. No. 4,897,255), protein kinase inhibitors (e.g., staurosporin), stimulators of the production or activation of TGF-beta, including **tamoxifen** and functional equivalents or derivatives thereof, TGF-beta or functional equivalents, derivatives or analogs thereof, taxol or analogs thereof (e.g., taxotere),. . . e.g., cytokines (e.g., interleukins such as IL-1), growth factors, (e.g., PDGF, TGF-alpha or -beta, tumor necrosis factor, smooth muscle- and **endothelial**-derived growth factors, i.e., **endothelin**, FGF), homing receptors (e.g., for platelets or leukocytes), and extracellular matrix receptors (e.g., integrins). Representative examples of useful therapeutic agents. . .

DETD . . . as well as diminish smooth muscle cell proliferation following angioplasty. The organization or stabilization may stem from the blockage of **vascular smooth muscle** cell maturation in to a pathologically proliferating form.

DETD For the sustained release dosage form embodiments of the present invention, therapeutic agents preferably are those that inhibit **vascular smooth muscle** cell activity without

killing the cells (i.e., cytostatic therapeutic agents). Another way to define a cytostatic agent is a moiety. . . or more of the following capabilities: to inhibit DNA synthesis prior to protein synthesis inhibition or to inhibit migration of **vascular smooth muscle** cells into the intima. These therapeutic agents do not significantly inhibit protein synthesis (i.e., do not kill the target cells). . .

DETD **Vascular smooth muscle** binding proteins of the invention bind to targets on the surface of **vascular smooth muscle** cells. It will be recognized that specific targets, e.g., polypeptides or carbohydrates, proteoglycans and the like, that are associated with the cell membranes of **vascular smooth muscle** cells are useful for selecting (e.g., by cloning) or constructing (e.g., by genetic engineering or chemical synthesis) appropriately specific **vascular smooth muscle** binding proteins. Particularly useful "targets" are internalized by smooth muscle cells, e.g., as membrane constituent antigen turnover occurs in renewal.. . . endocytosis and the like. In a preferred embodiment, such a "target" is exemplified by chondroitin sulfate proteoglycans (CSPGs) synthesized by **vascular smooth muscle** cells and pericytes, and a discrete portion (termed an epitope herein) of the CSPG molecule having an apparent molecular weight. . . is a component of a larger 400 kD proteoglycan complex (14). In one presently preferred embodiment of the invention, a **vascular smooth muscle** binding protein is provided by NR-AN-01 monoclonal antibody (a subculture of NR-ML-05) that binds to an epitope in a **vascular smooth muscle** CSPG target molecule. The monoclonal antibody designated NR-ML-05 reportedly binds a 250 kD CSPG synthesized by melanoma cells (Morgan et. . . and functionally equivalent to, subclone NR-AN-01, disclosed herein. It will be recognized that NR-AN-01 is just one example of a **vascular smooth muscle** binding protein that specifically associates with the 400 kD CSPG target, and that other binding proteins associating with this target. . . human chimeric monoclonal antibodies have also been selected, as described herein, that specifically target to the 250 kD CSPG of **vascular smooth muscle** cells. The antibodies also appear to be internalized by the smooth muscle cells following binding to the cell membrane. Immunoreactivity. . . No. 4,879,225). In this disclosure and other human clinical studies, MAbs directed to the CSPG 250 kD antigen localized to **vascular smooth muscle** cells in vivo. Further, it will be recognized that the amino acid residues involved in the multi-point kinetic association of. . . molecular model for constructing functional equivalents, e.g., short polypeptides ("minimal polypeptides"), that have binding affinity for a CSPG synthesized by **vascular smooth muscle** cells and pericytes.

DETD . . . antibodies or fragments, for use in the practice of the invention have a binding affinity of >10.sup.4 liter/mole for the **vascular smooth muscle** 250 kD CSPG, and also the ability to be bound to and internalized by smooth muscle cells or pericytes.

DETD . . . to achieve the proper spacing for binding to the amino acids of, for example, an NR-AN-01 CSPG target synthesized by **vascular smooth muscle** cells or pericytes.

DETD . . . will be recognized that the inventors also contemplate the utility of human monoclonal antibodies or "humanized" murine antibody as a **vascular smooth muscle** binding protein in the therapeutic conjugates of their invention. For example, murine monoclonal antibody may be "chimerized" by genetically recombining. . . residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics.

Humanized **vascular smooth muscle** binding partners will be recognized to have the advantage of decreasing the immunoreactivity of the antibody or polypeptide in the. . .

DETD . . . release dosage forms of the present invention are those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the **vascular smooth muscle** cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and. . .

DETD Representative "coupling" methods for linking the therapeutic agent through covalent or non-covalent bonds to the **vascular smooth muscle** binding protein include chemical cross-linkers and heterobifunctional cross-linking compounds (i.e., "linkers") that react to form a bond between reactive groups. . . hydroxyl, amino, amido, or sulfhydryl groups) in a therapeutic agent and other reactive groups (of a similar nature) in the **vascular smooth muscle** binding protein. This bond may be, for example, a peptide bond, disulfide bond, thioester bond, amide bond, thioether bond, and. . . reference, is instructive of coupling methods that may be useful. In one presently preferred embodiment, the therapeutic conjugate contains a **vascular smooth muscle** binding protein coupled covalently to a trichothecene drug. In this case, the covalent bond of the linkage may be formed between one or more amino, sulfhydryl, or carboxyl groups of the **vascular smooth muscle** binding protein and a) the trichothecene itself; b) a trichothecene hemisuccinate carboxylic acid; c) a trichothecene hemisuccinate (HS) N-hydroxy succinimidate. . .

DETD The choice of coupling method will be influenced by the choice of **vascular smooth muscle** binding protein or peptide, interstitial matrix binding protein or peptide and therapeutic agent, and also by such physical properties as,. . .

DETD . . . result in increased smooth muscle in the intimal region of a traumatized vascular site, e.g., following angioplasty, e.g., pericytes and **vascular smooth muscle** cells. Aspects of the invention relate to therapeutic modalities in which the therapeutic conjugate of the invention is used to. . .

DETD . . . example, this therapeutically effective dosage is achieved by preparing 10 ml of a 200 .mu.g/ml therapeutic conjugate solution, wherein the **vascular smooth muscle** protein binding protein is NR-AN-01 and the therapeutic agent is Roridin A, a trichothecene drug. For treating vascular trauma, e.g.,. . . therapeutic conjugate according to the invention will be dependent on several factors, including, e.g.: a) the binding affinity of the **vascular smooth muscle** binding protein in the therapeutic conjugate; b) the atmospheric pressure applied during infusion; c) the time over which the therapeutic. . .

DETD . . . extracellularly is distributed within the relevant intracellular compartment; and (3) the therapeutic agent inhibits the desired cellular activity of the **vascular smooth muscle** cell, e.g., proliferation, migration,. increased cellular volume, matrix synthesis, cell contraction and the like described above.

DETD Advantageously, non-coupled **vascular smooth muscle** cell binding protein (e.g., free NR-AN-01 antibody) is administered prior to administration of the therapeutic agent conjugate or dosage form to provide a blocker of non-specific binding to cross-reactive sites. Blocking of such sites is important because **vascular smooth muscle** cell binding proteins will generally have some low level of cross-reactivity with cells in

tissues other than the desired smooth. . . the specific vascular site, e.g., by making more of the therapeutic conjugate available to the

cells. As an example, non-coupled **vascular smooth muscle** binding protein is administered from about 5 minutes to about 48 hours, most preferably from about 5 minutes to about. . . of

minimizing displacement of the therapeutic conjugate or dosage form while maximizing blocking of the non-specific cross-reactive sites. The non-coupled **vascular smooth muscle** cell binding protein is administered in an amount effective to blocking binding of a least a portion of the non-specific. . .

DETD In addition, a second irrelevant **vascular smooth muscle** cell binding protein may optionally be administered to a patient prior to administration of the therapeutic conjugate or dosage form. . .

DETD . . . therapeutic agent. The cytocidal conjugate includes a binding partner (such as a protein or peptide) capable of specifically localizing to **vascular smooth muscle** cells and an active agent capable of killing such cells. The cytocidal conjugate is administered, preferably intravenously or through any. . . events. This cellular destruction causes the release of mitogens and other metabolic events, which events generally lead, in turn, to **vascular smooth muscle** cell proliferation.

The sustained release anti-proliferative or anti-contractile dosage forms of the present invention are next administered, preferably through

an infusion catheter or any convenient dosage form therefor. The sustained release dosage form retards the **vascular smooth muscle** cell proliferation and/or migration and contraction, thereby maintaining luminal diameter. This treatment methodology constitutes a biological arteromyectomy useful in stenotic vessels resulting from **vascular smooth muscle** cell hyperplasia and the like.

DETD . . . hours (preferably 24 to 72), an effective amount of a, for example, Pseudomonas exotoxin-monoclonal antibody conjugate capable of localizing to **vascular smooth muscle** cells is locally administered (e.g., via a catheter during an angioplasty procedure); and

DETD . . . embodiment of this aspect of the present invention involves administration of a therapeutic agent capable of inhibiting the ability of **vascular smooth muscle** cells to contract. Exemplary agents useful in the practice of this aspect of the present invention are those capable of. . . occur directly or indirectly through, for example, inhibition of calcium modulation, phosphorylation or other metabolic pathways implicated in contraction of

vascular smooth muscle cells.

DETD Cytochalasins are exemplary therapeutic agents capable of generating a biological stenting effect on **vascular smooth muscle** cells. Cytochalasins are thought to inhibit both migration and contraction of **vascular smooth muscle** cells by interacting with actin. The cytochalasins interact with the ends of filamentous actin to inhibit the elongation of

the. . . filaments. Low doses of cytochalasins (e.g., cytochalasin B) also disrupt microfilament networks of actin. In vitro data indicate that after **vascular smooth muscle** cells clear cytochalasin B, the cells regenerate enough polymerized actin to resume migration within about 24 hours. In vivo assessments reveal that **vascular smooth muscle** cells regain vascular tone within 2 to 4 days. It is during this recuperative period that the lumen diameter fixation. . .

DETD Inhibition of **vascular smooth muscle** cell migration (from the tunica media to the intima) has been demonstrated in

the same dose range (Example 11); however,. . . sustained exposure of
the vessel to the therapeutic agent is preferable in order to maximize these anti-migratory effects. If the **vascular smooth muscle** cells cannot migrate into the intima, they cannot proliferate there. Should **vascular smooth muscle** cells migrate to the intima, a subsequently administered anti-proliferative sustained release dosage form inhibits the intimal proliferation. As a result,. . .

DETD (ii) inhibits target cell proliferation (e.g., following 5 minute and 24
hour exposure to the agent, in vitro **vascular smooth muscle** tissue cultures demonstrate a level of inhibition of .sup.3 H-thymidine uptake and, preferably, display relatively less inhibition of .sup.3 H-leucine. . .

DETD . . . or more of the preceding attributes, the agent is subjected to a second testing protocol that involves longer exposure of **vascular smooth muscle** cells to the therapeutic agent.

DETD (i) upon long term (e.g., 5 days) exposure, the agent produces the same or similar in vitro effect on **vascular smooth muscle** tissue culture DNA synthesis and protein synthesis, as described above for the 5 minute and 24 hour exposures; and

DETD . . . pig femoral artery model. Preferably, such agents demonstrate a
50% or greater inhibition of cell proliferation in the tunica media **vascular smooth muscle** cells, as indicated by a 1 hour "BRDU flash labeling" prior to tissue collection and histological evaluation. If an agent. . . to permit intravenous administration to achieve the 50% inhibition, or if the agent is amenable to local delivery to the **vascular smooth muscle** cells with sustained release at an effective anti-proliferative dose. Sustained release agents are evaluated in a sustained release dosage form. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of
smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 15.
This mechanism is believed to constitute a portion of the mechanism that
maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels. The pathway has been elucidated by the inventors of a patent application. . .

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the
activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. The apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation
of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 16.
Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from

healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7+/-0.06 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 16). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 16. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular**

smooth muscle cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 15 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 16 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta, TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued lower dose delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD . . . involve the administration of taxol or analogs thereof in soluble or sustained release dosage form. Taxol is believed to stabilize **vascular smooth muscle** cells against division by binding to microtubules and inhibiting the organization and ordering of the microtubule network. Cell migration may. . .

DETD Binding to **Vascular Smooth Muscle** Cells In the Blood Vessel Wall In Vivo

DETD . . . (FIG. 1A and FIG. 1B). This photomicrograph (FIG. 1B) demonstrates the ability of the MAb to specifically bind to human **vascular smooth muscle** in vivo, and to be internalized by the cells and remain in the cells for extended periods.

DETD . . . conducted to determine the binding kinetics of a smooth muscle binding protein with a Ka of >10.sup.9 liter/mole. Because human **vascular smooth muscle** cells grow slowly in culture, and baboon smooth muscle cells were found to express the human CSPG cell surface marker,. . .

DETD . . . to determine, in a domestic pig model system, the infusion conditions suitable for delivery of a therapeutic conjugate to the **vascular smooth muscle** cells in carotid and femoral arteries. Delivery conditions were monitored by evaluating the penetration of the therapeutic conjugate into the vascular wall, and specific binding of the therapeutic conjugate to the **vascular smooth muscle** cells in the vessel wall.

DETD . . . wall of swine coronary and femoral arteries 3-5 days after surgery, and the NR-AN-01 appeared to be associated only with **vascular smooth muscle** cells. These findings suggest that NR-AN-01 is capable of specifically binding to its target antigen in vivo.

DETD Inhibition of **Vascular Smooth Muscle** Cells In Vivo

DETD . . . response to vascular trauma, including restenosis following angioplasty. Domestic pigs were used to study the effects of NR-AN-01 (i.e., termed **vascular smooth muscle** binding protein or simply VSMBP in these studies; and therapeutic conjugates with Roridin A are termed VSMBP - RA). The. . .

DETD . . . human coronary arteries. The test protocol was designed as an initial in vivo screening of intra-arteriolar, site specific, catheter administered, **vascular smooth muscle** binding protein (VSMBP) conjugates. Toxicity of free drug was also evaluated, i.e., for pathobiological effects on arteriolar smooth muscle cells.. . .

DETD . . . and stained with H&E, Massons Trichrome and Movats Pentachrome for morphological studies. Sections were also used for immunohistological staining of **vascular smooth muscle.**

DETD **Vascular Smooth Muscle** Cell In Vitro DNA and Protein Synthesis Inhibition

DETD The ability of various therapeutic agents to inhibit DNA synthesis and protein synthesis in **vascular smooth muscle** cells was tested. .sup.3 H-leucine and .sup.3 H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

DETD 5 minute exposure; .sup.3 H-leucine uptake: **Vascular smooth muscle** cells at 40,000 cells/ml were seeded in sterile 24 well plates at 1 ml/well. The plates were incubated overnight at. . . Co.sub.2, 95% air in a humidified atmosphere (saturation). Log dilutions of the therapeutic agent of interest were incubated with the **vascular smooth muscle** cells for 5 minutes or 24 hours. Samples of the therapeutic agents were diluted in DMEM:F-12 medium (Whittaker Bioproducts, Walkersville,. . .

DETD 5 minute exposure; .sup.3 H-thymidine uptake: **Vascular smooth muscle** cells were incubated in complete medium with 5% FBS (Gibco) overnight at 37.degree. C. in a humidified, 5% CO.sub.2 environment. . .

DETD Morphological Cytotoxicity Evaluation-Pulsed Exposure: **Vascular smooth muscle** cells were seeded at 4.0.times.10.sup.4 cells/ml medium/well on a commercially prepared four well slide (Nunc, Inc., Naperville, Ill.). Enough slides. . .

DETD . . . the practice of sustained release dosage form embodiments of the present invention. More specifically, the compounds inhibited the ability of **vascular smooth muscle** cells to

undergo DNA synthesis in the presence of 5% FBS to a greater extent than they inhibited protein synthesis of **vascular smooth muscle** cells. The protein and DNA synthesis inhibitory effects of suramin, staurosporin, nitroglycerin and cytochalasin B during a 5 minute and. . .

DETD Specific Binding and Internalization of Targeted Particles by **Vascular Smooth Muscle** Cells

DETD The ability of **vascular smooth muscle** cells to bind and internalize particles coated with binding protein or peptide was demonstrated with monoclonal antibody (NR-AN-01) coated gold beads both in vitro and in vivo. The **vascular smooth muscle** cell tissue cultures (BO54), an antigen positive control cell line (A375) and an antigen negative control cell line (HT29) were. . .

DETD . . . gold beads devoid of NR-AN-01 to surface mucin produced by HT29 cells was observed, resulting in modest non-specific internalization thereof. **Vascular smooth muscle** cell uptake of NR-AN-01 targeted gold beads was highly specific in cell suspension cultures.

DETD The targeted gold bead **vascular smooth muscle** cell surface binding, internalization and lysosome concentration was observed in vivo as well. NR-AN-01 coated gold beads were infused via. . . pig femoral artery immediately following balloon trauma. The bead internalization rate varied with the degree of damage sustained by the **vascular smooth muscle** cell during the balloon trauma. Cells with minimal or no damage rapidly internalized the particles by endocytosis and phagocytosis, concentrating. . .

DETD **Vascular Smooth Muscle** In Vitro DNA and Protein Synthesis Inhibition By Staurosporin and Cytochalasin

DETD The ability of staurosporin and cytochalasin to inhibit in vitro DNA and protein synthesis in **vascular smooth muscle** cells was tested. .sup.3 H-leucine and .sup.3 H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

DETD **Vascular smooth muscle** cells at 40,000-50,000 cells/ml were seeded and processed as described in Example 8, "5 minute exposure; .sup.3 H-leucine uptake." Log. . . ml/well of each therapeutic agent dilution was added in quadruplicate wells, and the agent of interest was incubated with the **vascular smooth muscle** cells for 5 min at room temperature in a sterile ventilated hood. Following therapeutic agent incubation, the wells were subsequently. . .

DETD 5 Minute Exposure; DNA Synthesis Assay: **Vascular smooth muscle** (BO54) cells were seeded and processed in 24 well plates, as described above under "5 Minute Exposure: Protein Synthesis Assay.". . .

DETD 24 and 120 Hour Exposure: Protein Synthesis Assay: **Vascular smooth muscle** (BO54) cells at 20,000 cells/ml were seeded in sterile 24 well plates and incubated in complete medium (1 ml/well) overnight. . .

DETD **Vascular Smooth Muscle** Cell Migration Inhibition

DETD **Vascular smooth muscle** cells (BO54) were derived from explants of baboon aortic smooth muscle, as described in Example 10. The cells were grown in flat bottom, six well tissue culture plates, which hold about 5 ml of medium. The **vascular smooth muscle** cells were plated at 200,000 cells/well and placed at 37.degree. C. in a humidified 5% CO.sub.2 incubator for

hours.. . .

DETD . . . indicates Migration Grade, wherein -=no migration; +1=minimal; +2=mild; +3=moderate; and +4=marked (maximum density; limit of cell contact inhibition) migration of **vascular smooth muscle** cells into the cleared area adjacent to the scratch. In this Table, "T" denotes a morphological Toxicity Grade, wherein -=no.

.

.

DETD The data indicate that cytochalasin B inhibits the migration (+1 to +2) of **vascular smooth muscle** cells into the cleared area adjacent to the scratch at a dose of 0.1 .mu.g/ml with only minimal (- to. . .

DETD TABLE 4

SCRATCH-MIGRATION ASSAY: INHIBITION OF **VASCULAR SMOOTH MUSCLE** CELL MIGRATION BY CYTOCHALASIN B

Continuous Exposure					7-day Recovery Post Exposure				
Dosage .mu.g/mL					Dosage .mu.g/mL				
	Control					Control			
Day	0.0	0.01	0.1	1.0	0.0.	.	.		

DETD Therapeutic Agent Cytotoxic Effects on **Vascular Smooth Muscle** Cells--Pulse and Continuous Exposure

DETD **Vascular smooth muscle** cells were exposed to a therapeutic agent in one of two exposure formats:

DETD In Vivo BRDU Assay: Inhibition of **Vascular Smooth Muscle** Cell Proliferation

DETD BRDU assay: In vivo **vascular smooth muscle** proliferation was quantitated by measuring incorporation of the base analog 5-bromo-2'-deoxyuridine (BRDU, available from Sigma Chemical Co.) into DNA during. . .

DETD . . . uptake relative to a PBS control; however, cytochalasin B and staurosporin inhibited BRDU uptake (i.e., cell proliferation) without killing the **vascular smooth muscle** cells. The number of **vascular smooth muscle** cells labeled with BRDU was assigned a grade at 400.times. magnification as follows:

DETD . . . from 0.1 .mu.g/ml (.apprxeq.140% increase) to 1.0 .mu.g/ml (FIG. 14). The 10 .mu.g/ml dose appeared to be toxic to the **vascular smooth muscle** cells (data not shown). The subthreshold dose (0.01 .mu.g/ml) and negative control (PBS) exhibited a .+-..apprxeq.20% change in luminal area.. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation

DETD Cell culture, DNA synthesis assay and cell counting. Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD RNA Preparation and Northern Analysis. Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD Results. **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614+/-1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-betal mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

CLM What is claimed is:

. . . functional analog of cytochalasin B in an amount and for a period of time effective to inhibit the contraction of **vascular smooth muscle** cells while not eliminating their ability to secrete extracellular matrix.

. . . cytoskeletal inhibitor in an amount and for a period of time effective to inhibit the contraction or migration of the **vascular smooth muscle** cells.

L7 ANSWER 10 OF 19 USPATFULL

AN 1998:154312 USPATFULL

TI Prevention and treatment of pathologies associated with abnormally proliferative smooth muscle cells

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PI US 5847007 19981208

AI US 1994-242161 19940512 (8)

RLI Continuation-in-part of Ser. No. US 1993-61714, filed on 13 May 1993, now abandoned

DT Utility

EXNAM Primary Examiner: Henley, III, Raymond

LREP Schwegman, Lundberg, Woessner & Kluth, P.A.

CLMN Number of Claims: 16

ECL Exemplary Claim: 1

DRWN 2 Drawing Figure(s); 2 Drawing Page(s)

LN.CNT 2429

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB . . . dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation and/or migration of **vascular smooth muscle** cells over time. Further provided is a method for determining TGF-beta in vitro, thereby identifying a patient at risk for. . .

SUMM . . . to the prevention and treatment of conditions characterized by abnormal smooth muscle cell proliferation. More specifically, mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation and agents that impact those mechanisms are discussed.

SUMM . . . cell proliferation. It would be highly advantageous to develop new compositions or methods for inhibiting stenosis due to proliferation of **vascular smooth muscle** cells following,

for example, traumatic injury to vessels rendered during vascular surgery.

SUMM . . . a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .

DRWD FIGS. 1 and 2 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.

DETD . . . with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a **vascular smooth muscle** cell or pericyte.

DETD . . . trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine which is capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell proliferation. Functional equivalents and derivatives of the aforementioned chemical compound are also included within the scope of the term. . .

DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta.

DETD TGF-beta activators or production stimulators of the invention are useful for inhibiting the pathological proliferation of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 1. This mechanism is believed to constitute a portion of the mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels.

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. The apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 2. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in

a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7+/-0.6 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing

the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 (a concentration resulting in 50% inhibition) at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 1 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 2 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that are. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued lower dose delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD Human **vascular smooth muscle** cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat (doubling time. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation

DETD Cell culture, DNA synthesis assay and cell counting. Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as

described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, MA). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD RNA Preparation and Northern Analysis. Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD Results. **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614+/-1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-betal mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little MRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Materials. Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. **Tamoxifen** (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were purchased from Flow Laboratories. 6-[.sup.3. . . factor and insulin-like growth facter 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Antiotensin II and **endothelin** 1 were obtained from Sigma and dissolved in sterile MilliQ water. TGF-beta (0.5 .mu.g, lyophilized solid) was purchased from Peninsula,. . .

L7 ANSWER 11 OF 19 USPATFULL

AN 1998:72634 USPATFULL

TI Prevention and treatment of cardiovascular pathologies

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PI US 5770609 19980623

AI US 1995-486334 19950607 (8)

RLI Continuation-in-part of Ser. No. US 1994-242161, filed on 12 May 1994 which is a continuation-in-part of Ser. No. US 1993-61714, filed on 13 May 1993, now abandoned And a continuation-in-part of Ser. No. US 1994-241844, filed on 12 May 1994 which is a continuation-in-part of Ser. No. US 1993-62451, filed on 13 May 1993, now abandoned which is a continuation-in-part of Ser. No. US 1993-11669, filed on 28 Jan 1993, now abandoned

DT Utility

EXNAM Primary Examiner: Henley, III, Raymond

LREP Schwegman, Lundberg, Woessner & Kluth, P.A.

CLMN Number of Claims: 56

ECL Exemplary Claim: 1

DRWN 2 Drawing Figure(s); 2 Drawing Page(s)
LN.CNT 4318
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
SUMM . . . can be considered to occur in five overlapping stages such as migration, lipid accumulation, recruitment of inflammatory cells, proliferation of **vascular smooth muscle** cells, and extracellular matrix deposition. Each of these processes can be shown to occur in man and in animal models. . .
SUMM The administered compound of formula (I) can act on **vascular smooth muscle** cells (VSMC) to inhibit the pathological activity of these smooth muscle cells and can inhibit lipid proliferative lesions. Preferably, the. . . comprises treatment of atherosclerosis, wherein the compound of formula (I), such as idoxifene or idoxifene salt, inhibits lipid accumulation by **vascular smooth muscle** cells and/or stabilizes an arterial lesion associated with atherosclerosis, i.e., increases plaque stability, to prevent rupture or growth of the. . .
SUMM A further aspect of the invention is a method comprising inhibiting **vascular smooth muscle** cell proliferation associated with procedural vascular trauma due to organ transplantation, vascular surgery, angioplasty, shunt placement, stent placement or vascular. . .
SUMM Yet a further aspect of the invention provides a method comprising inhibiting non-aortal **vascular smooth muscle** cell proliferation associated with procedural vascular trauma comprising administering an effective cytostatic antiproliferative amount of tamoxifen or a structural analog. . .
SUMM . . . of the present invention is a method for identifying a compound which is a TGF-beta activator or production stimulator. Human **vascular smooth muscle** cells (hVSMC) are cultured with an amount of the compound effective to reduce the normal rate of hVSMC proliferation, due. . . with an amount of an antibody which neutralizes TGF-beta activity. The method can also include the culture of rat aortic **vascular smooth muscle** cells (rVSMC) with an amount of the same compound effective to reduce the normal rate of proliferation of rVSMC, due. . .
SUMM . . . in the practice of the present invention to prevent or treat other conditions characterized by inappropriate or pathological activity of **vascular smooth muscle** cells. Such TGF-beta activators and production stimulators inhibit abnormal activity of **vascular smooth muscle** cells. Preferred compounds of formula (I) include those wherein Z is a covalent bond, Y is H, R.sup.3 is ClCH.sub.2. . .
DRWD FIGS. 1 and 2 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.
DETD . . . with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a **vascular smooth muscle** cell or pericyte.
DETD . . . thereof, which are capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell activity. Isomers and derivatives of the aforementioned chemical compound are also included within the scope of the term "tamoxifen". . .
DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta. "TGF-beta activator" includes moieties capable of. . .

DETD TGF-beta activators or production stimulators of the invention are useful for inhibiting the pathological proliferation of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .

DETD . . . a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 1. TGF-beta is believed to contribute to the inhibitory mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels.

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. An apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 2. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 .mu.M Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth**

muscle cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7+/-0.6 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 (a concentration resulting in 50% inhibition) at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated,

and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 1 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 2 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that are. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD . . . dosage forms involving sustained release of the TGF-beta activator or production stimulator to target cells. Preferably, the target cells are **vascular smooth muscle** cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that. . . the dosage form. Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting **vascular smooth muscle** cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, formation of lipid proliferative lesions, contraction, migration or the like) but does not kill the cell and, optionally, a **vascular smooth muscle** cell binding protein. Sustained released dosage forms for systemic administration as well as for local administration are also employed in.

. .

DETD . . . affect the rate and duration of the drug release required to achieve the cytostatic dosing which has been demonstrated in **vascular smooth muscle** cell tissue culture experiments. Different types of devices may require different periods of therapeutic drug release. For example, the use. . .

DETD Human **vascular smooth muscle** cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat. Medium conditioned. . .

DETD . . . is a TGF-beta activator or TGF-beta production stimulator, an agent or mixture of agents is first tested on rat aortic **vascular smooth muscle** cells (rVSMCs) for their ability to stimulate the production of active TGF-.beta. in the culture medium as originally described for. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production

and Activation
DETD Cell culture, DNA synthesis assay and cell counting. Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .
DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.
DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .
DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.
DETD RNA Preparation and Northern Analysis. Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .
DETD Results. **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM +10%. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .
DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .
DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614+/-1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.
DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether

tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-beta mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Materials. Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. **Tamoxifen** (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were purchased from Flow Laboratories. 6-[.sup.3. . . factor and insulin-like growth factor 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Antiotensin II and **endothelin** 1 were obtained from Sigma and dissolved in sterile MilliQ water. TGF-beta (0.5 .mu.g, lyophilized solid) was purchased from Peninsula,. . .

DETD TABLE 8

Mitogenic indices of human serum and plasma on human **vascular smooth muscle** cells

Donor	Mitogenic index Serum	Plasma
B	45	0.7
H	52	1.4
C	60	0.9
D	65	1.0
A	83	1.2

DMEM containing 5% serum or. . .

CLM What is claimed is:

13. A therapeutic method comprising inhibiting **vascular smooth muscle** cell proliferation associated with

procedural vascular trauma comprising administration to a mammal subjected to said procedural trauma an effective antiproliferative. .

. 51. A therapeutic method comprising inhibiting **vascular smooth muscle** cell proliferation comprising administering to a mammal an effective cytostatic antiproliferative amount of a compound of formula (I): ##STR6## wherein. . .

L7 ANSWER 12 OF 19 USPATFULL
AN 1998:33947 USPATFULL
TI Therapeutic inhibitor of **vascular smooth muscle** cells
IN Kunz, Lawrence L., Redmond, WA, United States
Klein, Richard A., Lynnwood, WA, United States
Reno, John M., Brier, WA, United States
Grainger, David J., Cambridge, United Kingdom
Metcalfe, James C., Cambridge, United Kingdom
Weissberg, Peter L., Cambridge, United Kingdom
Anderson, Peter G., Brimingham, AL, United States
PA NeoRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 5733925 19980331
AI US 1996-738733 19961028 (8)
RLI Division of Ser. No. US 1995-450793, filed on 25 May 1995 which is a continuation of Ser. No. US 1993-62451, filed on 13 May 1993, now abandoned which is a continuation-in-part of Ser. No. US 1993-11669, filed on 28 Jan 1993, now abandoned
DT Utility
EXNAM Primary Examiner: Barts, Samuel
LREP Schwegman, Lundberg, Woessner & Kluth, P.A.
CLMN Number of Claims: 28
ECL Exemplary Claim: 1
DRWN 29 Drawing Figure(s); 21 Drawing Page(s)
LN.CNT 4753
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
TI Therapeutic inhibitor of **vascular smooth muscle** cells
AB . . . disease in a mammalian host, comprising administering to the host a therapeutically effective dosage of a therapeutic conjugate containing a **vascular smooth muscle** binding protein that associates in a specific manner with a cell surface of the **vascular smooth muscle** cell, coupled to a therapeutic agent dosage form that inhibits a cellular activity of the muscle cell. Methods are also provided for the direct and/or targeted delivery of therapeutic agents to **vascular smooth muscle** cells that cause a dilation and fixation of the vascular lumen by inhibiting smooth muscle cell contraction, thereby constituting a biological stent. Also discussed are mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation, agents that impact those mechanisms and protocols for the use of those agents.
SUMM . . . smooth muscle proteins is also described. The invention also relates to the direct or targeted delivery of therapeutic agents to **vascular smooth muscle** cells that results in dilation and fixation of the vascular lumen (biological stenting effect). Combined administration of a cytocidal conjugate and a sustained release dosage form of a **vascular smooth muscle** cell inhibitor is also disclosed. Mechanisms for in vivo **vascular smooth muscle** cell proliferation modulation, agents that impact those mechanisms and protocols for the use of those agents are discussed.
SUMM . . . smooth muscle cell proliferation. It would be highly advantageous to develop new methods for inhibiting stenosis due to proliferation of **vascular smooth muscle** cells following traumatic injury to vessels such as occurs during vascular surgery. In addition, delivery of compounds that produce

inhibitory effects of extended duration to the **vascular smooth muscle** cells would be advantageous. Local administration of such sustained release compounds would also be useful in the treatment of other. . .

SUMM In one aspect of the invention, new therapeutic methods and therapeutic conjugates are provided for inhibiting **vascular smooth muscle** cells in a mammalian host. The therapeutic conjugates contain a **vascular smooth muscle** binding protein or peptide that binds in a specific manner to the cell membranes of a **vascular smooth muscle** cell or an interstitial matrix binding protein/peptide that binds in a specific manner to interstitial matrix (e.g., collagen) of the. . . after angioplasty or other vascular surgical procedures. The therapeutic conjugates of the invention achieve these advantageous effects by associating with **vascular smooth muscle** cells and pericytes, which may transform into smooth muscle cells. The therapeutic conjugate may contain: (1) therapeutic agents that alter.

.

. the like) are also contemplated for use in accordance with the present invention. Other aspects of the invention relate to **vascular smooth muscle** binding proteins that specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a **vascular smooth muscle** cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments the **vascular smooth muscle** binding protein binds to a CSPG target on the cell surface with an association constant of at least 10.sup.-4 M. In another preferred embodiment, the **vascular smooth muscle** binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal. . .

SUMM . . . therapeutic methods and therapeutic dosage forms involving sustained release of therapeutic agent to target cells. Preferably, the target cells are **vascular smooth muscle** cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that. . . the dosage form. Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting **vascular smooth muscle** cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, contraction, migration or the like) but does not kill the cell and, optionally, a **vascular smooth muscle** cell binding protein. Also, the methods and dosage forms of this aspect of the present invention are useful for inhibiting. .

.

SUMM . . . therapeutically significant target cell activity without killing the target cell, or target cell killing activity. For treatment of restenosis of **vascular smooth muscle** cells, useful therapeutic agents inhibit target cell activity (e.g., proliferation or migration) without killing the target cells. Preferred therapeutic moieties. . .

SUMM . . . to a relevant target cell population by a binding protein or peptide. Preferred binding proteins/peptides of the present invention are **vascular smooth muscle** cell binding protein, tumor cell binding protein and immune system effector cell binding protein. Preferred **vascular smooth muscle** cell binding proteins specifically associate with a chondroitin sulfate proteoglycan (CSPG) expressed on the membranes of a **vascular smooth muscle** cell, and in a preferred embodiment this CSPG has a molecular weight of about 250 kDaltons. In preferred embodiments, the **vascular smooth muscle** binding protein binds to a CSPG target on the cell surface with an association constant of at least 10.sup.-4 M. In other preferred embodiments, the **vascular smooth**

muscle binding protein contains a sequence of amino acids found in the Fab, Fv or CDR (complementarity determining regions) of monoclonal. . . in this embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells.

Preferred binding peptides of this type are specifically associated with collagen, reticulum fibers or other intercellular matrix compounds. Preferred. . .

SUMM . . . involving administration of free (i.e., non-targeted or non-binding partner associated) therapeutic agent to target cells. Preferably, the target cells are **vascular smooth muscle** cells and the therapeutic agent is an inhibitor of **vascular smooth muscle** cell contraction, allowing the normal hydrostatic pressure to dilate the vascular lumen. Such contraction inhibition may be achieved by actin. . . which is preferably achievable and sustainable at a lower dose level than that necessary to inhibit protein synthesis. Consequently, the **vascular smooth muscle** cells synthesize protein required to repair minor cell trauma and secrete interstitial matrix, thereby facilitating the fixation of the vascular. . . post-procedural angiogram. Cytochalasins (which inhibit the polymerization of G- to F-actin which, in turn, inhibits the migration and contraction of **vascular smooth muscle** cells) are the preferred therapeutic agents for use in this embodiment of the present invention. Free therapeutic agent protocols of. . . of stenosis after angioplasty or other vascular surgical procedures. Preferably, free therapeutic agent is administered directly or substantially directly to **vascular smooth muscle** tissue. Such administration is preferably effected by an infusion catheter, to achieve a 10.sup.-3 M to 10.sup.-12 M concentration of. . .

SUMM Another embodiment of the present invention incorporates administration of a cytocidal targeted conjugate to destroy proliferating **vascular smooth muscle** cells involved in vascular stenosis. The mitogenic agents released after this biological arteromyectomy are prevented from stimulating the remaining viable **vascular smooth muscle** cells to proliferate and restenose the vessel by administration of the anti-contraction (anti-migration) or anti-proliferative sustained release agents of the. . .

SUMM . . . Such dosage forms are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .

DRWD FIG. 1A is a photomicrograph of **vascular smooth muscle** cells of a 24-year old male patient.

DRWD FIG. 1B is a photomicrograph of **vascular smooth muscle** cells in an artery of a 24-year-old male patient with **vascular smooth muscle** binding protein bound to the cell surface and membrane. The patient received the **vascular smooth muscle** binding protein by i.v. administration 4 days before the arterial tissue was prepared for histology.

DRWD FIG. 2 depicts a first scheme for chemical coupling of a therapeutic agent to a **vascular smooth muscle** binding protein.

DRWD FIG. 3 depicts a second scheme for chemical coupling of a therapeutic agent to a **vascular smooth muscle** binding protein.

DRWD FIG. 4A graphically depicts experimental data showing rapid binding of **vascular smooth muscle** binding protein to marker-positive test cells in vitro.

DRWD FIG. 4B graphically depicts experimental data showing rapid binding of **vascular smooth muscle** binding protein to **vascular smooth muscle** cells in vitro.

DRWD . . . data showing undesirable cytotoxicity of even low levels of therapeutic conjugate (i.e., RA-NR-AN-01), and the free RA therapeutic agent, when **vascular smooth muscle** cells were treated for 24 hours in vitro.

DRWD . . . RA therapeutic agent was non-specifically cytotoxic, the RA-NR-AN-01 therapeutic conjugate did not exert long-lasting inhibitory effects on cellular activity in **vascular smooth muscle** cells, as evidenced by metabolic activity in BO54 cells that were allowed a 48 hour recovery period prior to testing.

DRWD FIG. 10A graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of suramin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10B graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of staurosporin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10C graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of nitroglycerin with respect to **vascular smooth muscle** cells.

DRWD FIG. 10D graphically depicts experimental data comparing protein synthesis and DNA synthesis inhibition capability of cytochalasin B with respect to **vascular smooth muscle** cells.

DRWD FIGS. 15 and 16 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.

DETD "Therapeutic conjugate" means a **vascular smooth muscle** or an interstitial matrix binding protein coupled (e.g., optionally through a linker) to a therapeutic agent.

DETD . . . the conjugate aspects of the present invention to mean a molecule recognized in a specific manner by the matrix or **vascular smooth muscle** binding protein, e.g., an antigen, polypeptide antigen or cell surface carbohydrate (e.g., a glycolipid, glycoprotein, or proteoglycan) that is expressed on the cell surface membranes of a **vascular smooth muscle** cell or a matrix structure.

DETD . . . covalent or non-covalent chemical association (i.e., hydrophobic as through van der Waals forces or charge-charge interactions) of the matrix or **vascular smooth muscle** binding protein with the therapeutic agent. Due to the nature of the therapeutic agents employed, the binding proteins will normally. . .

DETD . . . transcription and translation with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., CSPG synthesized by a **vascular smooth muscle** cell or pericyte.

DETD "Cytochalasin" includes fungal metabolites exhibiting an inhibitory effect on target cellular metabolism, including prevention of contraction or migration of **vascular smooth muscle** cells. Preferably, cytochalasins inhibit the polymerization of monomeric actin (G-actin) to polymeric form (F-actin), thereby inhibiting cell functions requiring cytoplasmic. . .

DETD . . .)phenoxy]-N,N-dimethyl-ethylamine which is capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell proliferation. Evidence exists that tamoxifen also acts to stabilize or organize areas of smooth muscle cell trauma. This organization/stabilization. . .

DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved.

Functional equivalents of TGF-beta are, for example, moieties capable of. . .

DETD . . . negative immuno-staining with antibodies to cytokeratins (i.e., epithelial and fibroblast markers) and yon Willdebrand factor (i.e., an endothelial marker). Both **vascular smooth muscle** cells and pericytes are positive by immunostaining with the NR-AN-01 monoclonal antibody.

DETD The therapeutic conjugates and dosage forms of the invention are useful for inhibiting the activity of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .

DETD Therapeutic conjugates of the invention are obtained by coupling a **vascular smooth muscle** binding protein to a therapeutic agent. In the therapeutic conjugate, the **vascular smooth muscle** binding protein performs the function of targeting the therapeutic conjugate to **vascular smooth muscle** cells or pericytes, and the therapeutic agent performs the function of inhibiting the cellular activity of the smooth muscle cell. . .

DETD Nanoparticulate sustained release therapeutic dosage forms of preferred embodiments of the present invention are biodegradable and bind to the **vascular smooth muscle** cells and enter such cells primarily by endocytosis. The biodegradation of such nanoparticulates occurs over time (e.g., 10 to 21. . .

DETD Useful **vascular smooth muscle** binding protein is a polypeptide, peptidic, or mimetic compound (as described below) that is capable of binding to a target or marker on a surface component of an intact or disrupted **vascular smooth muscle** cell in such a manner that allows for either release of therapeutic agent extracellularly in the immediate interstitial matrix with. . . into an intracellular compartment of the entire targeted biodegradable moiety, permitting delivery of the therapeutic agent. Representative examples of useful **vascular smooth muscle** binding proteins includes antibodies (e.g., monoclonal and polyclonal affinity-purified antibodies, F(ab').sub.2, Fab', Fab, and Fv fragments and/or complementarity determining regions. . .

DETD . . . dosage form embodiment of the present invention include those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the **vascular smooth muscle** cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and. . .

DETD Therapeutic agents of the invention are selected to inhibit a cellular activity of a **vascular smooth muscle** cell, e.g., proliferation, migration, increase in cell volume, increase in extracellular matrix synthesis (e.g., collagens, proteoglycans, and the like), or. . . spindle fiber formation (e.g., a drug such as colchicine) and the like; or b) as an inhibitor of migration of **vascular smooth muscle** cells from the medial wall into the intima, e.g., an "anti-migratory agent" such as a cytochalasin; or c) as an. . .

DETD . . . et al., U.S. Pat. No. 4,897,255), protein kinase inhibitors (e.g., staurosporin), stimulators of the production or activation of TGF-beta, including **tamoxifen** and functional equivalents or derivatives thereof, TGF-beta or functional equivalents, derivatives or analogs thereof, taxol or analogs thereof (e.g., taxotere),. . . e.g., cytokines (e.g., interleukins such as IL-1), growth factors, (e.g., PDGF, TGF-alpha or -beta, tumor necrosis factor, smooth muscle- and **endothelial**-derived growth factors, i.e.,

endothelin, FGF), homing receptors (e.g., for platelets or leukocytes), and extracellular matrix receptors (e.g., integrins). Representative examples of useful therapeutic agents. . .

DETD . . . as well as diminish smooth muscle cell proliferation following angioplasty. The organization or stabilization may stem from the blockage of **vascular smooth muscle** cell maturation in to a pathologically proliferating form.

DETD For the sustained release dosage form embodiments of the present invention, therapeutic agents preferably are those that inhibit **vascular smooth muscle** cell activity without killing the cells (i.e., cytostatic therapeutic agents). Another way to define a cytostatic agent is a moiety. . . or more of the following capabilities: to inhibit DNA synthesis prior to protein synthesis inhibition or to inhibit migration of **vascular smooth muscle** cells into the intima. These therapeutic agents do not significantly inhibit protein synthesis (i.e., do not kill the target cells). . .

DETD **Vascular smooth muscle** binding proteins of the invention bind to targets on the surface of **vascular smooth muscle** cells. It will be recognized that specific targets, e.g., polypeptides or carbohydrates, proteoglycans and the like, that are associated with the cell membranes of **vascular smooth muscle** cells are useful for selecting (e.g., by cloning) or constructing (e.g., by genetic engineering or chemical synthesis) appropriately specific **vascular smooth muscle** binding proteins. Particularly useful "targets" are internalized by smooth muscle cells, e.g., as membrane constituent antigen turnover occurs in renewal.. . . endocytosis and the like. In a preferred embodiment, such a "target" is exemplified by chondroitin sulfate proteoglycans (CSPGs) synthesized by **vascular smooth muscle** cells and pericytes, and a discrete portion (termed an epitope herein) of the CSPG molecule having an apparent molecular weight. . . is a component of a larger 400 kD proteoglycan complex (14). In one presently preferred embodiment of the invention, a **vascular smooth muscle** binding protein is provided by NR-AN-01 monoclonal antibody (a subculture of NR-ML-05) that binds to an epitope in a **vascular smooth muscle** CSPG target molecule. The monoclonal antibody designated NR-ML-05 reportedly binds a 250 kD CSPG synthesized by melanoma cells (Morgan et. . . and functionally equivalent to, subclone NR-AN-01, disclosed herein. It will be recognized that NR-AN-01 is just one example of a **vascular smooth muscle** binding protein that specifically associates with the 400 kD CSPG target, and that other binding proteins associating with this target. . . human chimeric monoclonal antibodies have also been selected, as described herein, that specifically target to the 250 kD CSPG of **vascular smooth muscle** cells. The antibodies also appear to be internalized by the smooth muscle cells following binding to the cell membrane. Immunoreactivity. . . No. 4,879,225). In this disclosure and other human clinical studies, MAbs directed to the CSPG 250 kD antigen localized to **vascular smooth muscle** cells in vivo. Further, it will be recognized that the amino acid residues involved in the multi-point kinetic association of. . . molecular model for constructing functional equivalents, e.g., short polypeptides ("minimal polypeptides"), that have binding affinity for a CSPG synthesized by **vascular smooth muscle** cells and pericytes.

DETD . . . antibodies or fragments, for use in the practice of the invention have a binding affinity of >10.sup.4 liter/mole for the **vascular smooth muscle** 250 kD CSPG, and also the ability to be bound to and internalized by smooth muscle cells or pericytes.

DETD . . . to achieve the proper spacing for binding to the amino acids

of, for example, an NR-AN-01 CSPG target synthesized by **vascular smooth muscle** cells or pericytes.

DETD . . . will be recognized that the inventors also contemplate the utility of human monoclonal antibodies or "humanized" murine antibody as a **vascular smooth muscle** binding protein in the therapeutic conjugates of their invention. For example, murine monoclonal antibody may be "chimerized" by genetically recombining. . . residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics. Humanized **vascular smooth muscle** binding partners will be recognized to have the advantage of decreasing the immunoreactivity of the antibody or polypeptide in the. . .

DETD . . . release dosage forms of the present invention are those that localize to intercellular stroma and matrix located between and among **vascular smooth muscle** cells. Such binding peptides deliver the therapeutic agent to the interstitial space between the target cells. The therapeutic agent is released into such interstitial spaces for subsequent uptake by the **vascular smooth muscle** cells. Preferred binding peptides of this type are associated with epitopes on collagen, extracellular glycoproteins such as tenascin, reticulum and. . .

DETD Representative "coupling" methods for linking the therapeutic agent through covalent or non-covalent bonds to the **vascular smooth muscle** binding protein include chemical cross-linkers and heterobifunctional cross-linking compounds (i.e., "linkers") that react to form a bond between reactive groups. . . hydroxyl, amino, amido, or sulfhydryl groups) in a therapeutic agent and other reactive groups (of a similar nature) in the **vascular smooth muscle** binding protein. This bond may be, for example, a peptide bond, disulfide bond, thioester bond, amide bond, thioether bond, and. . . reference, is instructive of coupling methods that may be useful. In one presently preferred embodiment, the therapeutic conjugate contains a **vascular smooth muscle** binding protein coupled covalently to a trichothecene drug. In this case, the covalent bond of the linkage may be formed between one or more amino, sulfhydryl, or carboxyl groups of the **vascular smooth muscle** binding protein and a) the trichothecene itself; b) a trichothecene hemisuccinate carboxylic acid; c) a trichothecene hemisuccinate (HS) N-hydroxy succinimidate. . .

DETD The choice of coupling method will be influenced by the choice of **vascular smooth muscle** binding protein or peptide, interstitial matrix binding protein or peptide and therapeutic agent, and also by such physical properties as,. . .

DETD . . . result in increased smooth muscle in the intimal region of a traumatized vascular site, e.g., following angioplasty, e.g., pericytes and **vascular smooth muscle** cells. Aspects of the invention relate to therapeutic modalities in which the therapeutic conjugate of the invention is used to. . .

DETD . . . example, this therapeutically effective dosage is achieved by preparing 10 ml of a 200 .mu.g/ml therapeutic conjugate solution, wherein the **vascular smooth muscle** protein binding protein is NR-AN-01 and the therapeutic agent is Roridin A, a trichothecene drug. For treating vascular trauma, e.g.,. . . therapeutic conjugate according to the invention will be dependent on several factors, including, e.g.: a) the binding affinity of the **vascular smooth muscle** binding protein in the therapeutic conjugate; b) the atmospheric pressure applied during infusion; c) the time over which the therapeutic. . .

DETD . . . extracellularly is distributed within the relevant intracellular compartment; and (3) the therapeutic agent inhibits the desired cellular activity of the **vascular smooth**

muscle cell, e.g., proliferation, migration, increased cellular volume, matrix synthesis, cell contraction and the like described above.

DETD Advantageously, non-coupled **vascular smooth muscle** cell binding protein (e.g., free NR-AN-01 antibody) is administered prior to administration of the therapeutic agent conjugate or dosage form to provide a blocker of non-specific binding to cross-reactive sites. Blocking of such sites is important because **vascular smooth muscle** cell binding proteins will generally have some low level of cross-reactivity with cells in tissues other than the desired smooth. . . the specific vascular site, e.g., by making more of the therapeutic conjugate available to the cells. As an example, non-coupled **vascular smooth muscle** binding protein is administered from about 5 minutes to about 48 hours, most preferably from about 5 minutes to about. . . of minimizing displacement of the therapeutic conjugate or dosage form while maximizing blocking of the non-specific cross-reactive sites. The non-coupled **vascular smooth muscle** cell binding protein is administered in an amount effective to blocking binding of a least a portion of the non-specific. . .

DETD In addition, a second irrelevant **vascular smooth muscle** cell binding protein may optionally be administered to a patient prior to administration of the therapeutic conjugate or dosage form. . .

DETD . . . therapeutic agent. The cytocidal conjugate includes a binding partner (such as a protein or peptide) capable of specifically localizing to **vascular smooth muscle** cells and an active agent capable of killing such cells. The cytocidal conjugate is administered, preferably intravenously or through any. . . events. This cellular destruction causes the release of mitogens and other metabolic events, which events generally lead, in turn, to **vascular smooth muscle** cell proliferation.

The sustained release anti-proliferative or anti-contractile dosage forms of the present invention are next administered, preferably through an infusion catheter or any convenient dosage form therefor. The sustained release dosage form retards the **vascular smooth muscle** cell proliferation and/or migration and contraction, thereby maintaining luminal diameter. This treatment methodology constitutes a biological arteromyectomy useful in stenotic vessels resulting from **vascular smooth muscle** cell hyperplasia and the like.

DETD . . . hours (preferably 24 to 72), an effective amount of a, for example, Pseudomonas exotoxin-monoclonal antibody conjugate capable of localizing to **vascular smooth muscle** cells is locally administered (e.g., via a catheter during an angioplasty procedure); and

DETD . . . embodiment of this aspect of the present invention involves administration of a therapeutic agent capable of inhibiting the ability of **vascular smooth muscle** cells to contract. Exemplary agents useful in the practice of this aspect of the present invention are those capable of. . . occur directly or indirectly through, for example, inhibition of calcium modulation, phosphorylation or other metabolic pathways implicated in contraction of **vascular smooth muscle** cells.

DETD Cytochalasins are exemplary therapeutic agents capable of generating a biological stenting effect on **vascular smooth muscle** cells. Cytochalasins are thought to inhibit both migration and contraction of **vascular smooth muscle** cells by interacting with actin. The cytochalasins interact with the ends of filamentous actin to inhibit the elongation of the. . . filaments. Low doses of cytochalasins (e.g., cytochalasin B)

also disrupt microfilament networks of actin. In vitro data indicate that after **vascular smooth muscle** cells clear cytochalasin B, the cells regenerate enough polymerized actin to resume migration within about 24 hours. In vivo assessments reveal that **vascular smooth muscle** cells regain vascular tone within 2 to 4 days. It is during this recuperative period that the lumen diameter fixation. . .

DETD Inhibition of **vascular smooth muscle** cell migration (from the tunica media to the intima) has been demonstrated in the same dose range (Example 11); however,. . . sustained exposure of the vessel to the therapeutic agent is preferable in order to maximize these anti-migratory effects. If the **vascular smooth muscle** cells cannot migrate into the intima, they cannot proliferate there. Should **vascular smooth muscle** cells migrate to the intima, a subsequently administered anti-proliferative sustained release dosage form inhibits the intimal proliferation. As a result,. . .

DETD (ii) inhibits target cell proliferation (e.g., following 5 minute and 24 hour exposure to the agent, in vitro **vascular smooth muscle** tissue cultures demonstrate a level of inhibition of .sup.3 H-thymidine uptake and, preferably, display relatively less inhibition of .sup.3 H-leucine. . .

DETD . . . or more of the preceding attributes, the agent is subjected to a second testing protocol that involves longer exposure of **vascular smooth muscle** cells to the therapeutic agent.

DETD (i) upon long term (e.g., 5 days) exposure, the agent produces the same or similar in vitro effect on **vascular smooth muscle** tissue culture DNA synthesis and protein synthesis, as described above for the 5 minute and 24 hour exposures; and

DETD . . . pig femoral artery model. Preferably, such agents demonstrate a 50% or greater inhibition of cell proliferation in the tunica media **vascular smooth muscle** cells, as indicated by a 1 hour "BRDU flash labeling" prior to tissue collection and histological evaluation. If an agent. . . to permit intravenous administration to achieve the 50% inhibition, or if the agent is amenable to local delivery to the **vascular smooth muscle** cells with sustained release at an effective anti-proliferative dose. Sustained release agents are evaluated in a sustained release dosage form. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 15. This mechanism is believed to constitute a portion of the mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels. The pathway has been elucidated by the inventors of a patent application. . .

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. The apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the

activation of the latent form of TGF-beta and facilitating proliferation
of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 16. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM) +10% fetal calf serum (FCS) as. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82.+-.4 hours to 47.+-.4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7.+-.0.06 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47.+-.3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82.+-.4 hours. . . doubling time for cultures of this experiment being 45.+-.6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 16). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation
of **vascular smooth muscle** cells, while

plasmin nullifies the growth stimulation of Lp(a). These results support
the theory that the mode of action of. . .
DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell
proliferation is set forth in detail in Example 16. The results of those
experiments are summarized below.
DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore
appears to be the result of an increase in the cell cycle time of nearly. . .
DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing
the time taken to traverse the G.sub.2 to M phase of the cell cycle.
DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.
DETD 5) **Vascular smooth muscle** cells produced
TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta
and increasing the proportion of the total TGF-beta which has been activated.
DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least
10-fold lower for **vascular smooth muscle**
cells than for adventitial fibroblasts.
DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with
the population doubling time in the presence of tamoxifen and Lp(a) being 42.+-.2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with
tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .
DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by
the pathway shown in FIG. 15 can be identified by a practitioner in the art by conducting. . .
DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by
the pathway shown in FIG. 16 can be identified by a practitioner in the art by conducting. . .
DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in
the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .
DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of
TGF-beta, TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation
caused by highly traumatic injuries or procedures such as angioplasty. Continued lower dose delivery to the traumatized site further protects

against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD . . . involve the administration of taxol or analogs thereof in soluble or sustained release dosage form. Taxol is believed to stabilize **vascular smooth muscle** cells against division by binding to microtubules and inhibiting the organization and ordering of the microtubule network. Cell migration may. . .

DETD Binding to **Vascular Smooth Muscle** Cells In the Blood Vessel Wall In Vivo

DETD . . . cells (FIG. 1A and FIG. 1B). This photomicrograph demonstrates the ability of the MAb to specifically bind to human **vascular smooth muscle** in vivo, and to be internalized by the cells and remain in the cells for extended periods.

DETD . . . conducted to determine the binding kinetics of a smooth muscle binding protein with a Ka of >10.sup.9 liter/mole. Because human **vascular smooth muscle** cells grow slowly in culture, and baboon smooth muscle cells were found to express the human CSPG cell surface marker,. . .

DETD . . . to determine, in a domestic pig model system, the infusion conditions suitable for delivery of a therapeutic conjugate to the **vascular smooth muscle** cells in carotid and femoral arteries. Delivery conditions were monitored by evaluating the penetration of the therapeutic conjugate into the vascular wall, and specific binding of the therapeutic conjugate to the **vascular smooth muscle** cells in the vessel wall.

DETD . . . wall of swine coronary and femoral arteries 3-5 days after surgery, and the NR-AN-01 appeared to be associated only with **vascular smooth muscle** cells. These findings suggest that NR-AN-01 is capable of specifically binding to its target antigen in vivo.

DETD Inhibition of **Vascular Smooth Muscle** Cells In Vivo

DETD . . . response to vascular trauma, including restenosis following angioplasty. Domestic pigs were used to study the effects of NR-AN-01 (i.e., termed **vascular smooth muscle** binding protein or simply VSMBP in these studies; and therapeutic conjugates with Roridin A are termed VSMBP-RA). The events which. . .

DETD . . . human coronary arteries. The test protocol was designed as an initial in vivo screening of intra-arteriolar, site specific, catheter administered, **vascular smooth muscle** binding protein (VSMBP) conjugates. Toxicity of free drug was also evaluated, i.e., for pathobiological effects on arteriolar smooth muscle cells.. . .

DETD . . . and stained with H&E, Massons Trichrome and Movats Pentachrome for morphological studies. Sections were also used for immunohistological staining of **vascular smooth muscle**.

DETD **Vascular Smooth Muscle** Cell In Vitro DNA and Protein Synthesis Inhibition

DETD The ability of various therapeutic agents to inhibit DNA synthesis and protein synthesis in **vascular smooth muscle** cells was tested. .sup.3 H-leucine and .sup.3 H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

DETD **Vascular smooth muscle** cells at 40,000 cells/ml were seeded in sterile 24 well plates at 1 ml/well. The plates were incubated overnight at. . . CO.sub.2, 95% air in a humidified atmosphere (saturation). Log dilutions of the therapeutic agent of interest were incubated with the **vascular smooth muscle** cells for 5 minutes or 24 hours. Samples of the therapeutic agents were diluted in DMEM:F-12 medium (Whittaker Bioproducts, Walkersville,. . .

DETD **Vascular smooth muscle** cells were

incubated in complete medium with 5% FBS (Gibco) overnight at 37.degree.
C. in a humidified, 5% CO.sub.2 environment. . .

DETD **Vascular smooth muscle** cells were seeded at 4.0.times.10.sup.4 cells/ml medium/well on a commercially prepared four well slide (Nunc, Inc., Naperville, Ill.). Enough slides. . .

DETD . . . the practice of sustained release dosage form embodiments of the present invention. More specifically, the compounds inhibited the ability of **vascular smooth muscle** cells to undergo DNA synthesis in the presence of 5% FBS to a greater extent than they inhibited protein synthesis of **vascular smooth muscle** cells. The protein and DNA synthesis inhibitory effects of suramin, staurosporin, nitroglycerin and cytochalasin B during a 5 minute and. . .

DETD Specific Binding and Internalization of Targeted Particles by **Vascular Smooth Muscle** Cells

DETD The ability of **vascular smooth muscle** cells to bind and internalize particles coated with binding protein or peptide was demonstrated with monoclonal antibody (NR-AN-01) coated gold beads both in vitro and in vivo. The **vascular smooth muscle** cell tissue cultures (BO54), an antigen positive control cell line (A375) and an antigen negative control cell line (HT29) were. . .

DETD . . . gold beads devoid of NR-AN-01 to surface mucin produced by HT29 cells was observed, resulting in modest non-specific internalization thereof. **Vascular smooth muscle** cell uptake of NR-AN-01 targeted gold beads was highly specific in cell suspension cultures.

DETD The targeted gold bead **vascular smooth muscle** cell surface binding, internalization and lysosome concentration was observed in vivo as well. NR-AN-01 coated gold beads were infused via. . . pig femoral artery immediately following balloon trauma. The bead internalization rate varied with the degree of damage sustained by the **vascular smooth muscle** cell during the balloon trauma. Cells with minimal or no damage rapidly internalized the particles by endocytosis and phagocytosis, concentrating. . .

DETD **Vascular Smooth Muscle** In Vitro DNA and Protein Synthesis Inhibition By Staurosporin and Cytochalasin

DETD The ability of staurosporin and cytochalasin to inhibit in vitro DNA and protein synthesis in **vascular smooth muscle** cells was tested. .sup.3 H-leucine and .sup.3 H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

DETD **Vascular smooth muscle** cells at 40,000-50,000 cells/ml were seeded and processed as described in Example 8, "5 minute exposure; .sup.3 H-leucine uptake." Log. . . ml/well of each therapeutic agent dilution was added in quadruplicate wells, and the agent of interest was incubated with the **vascular smooth muscle** cells for 5 min at room temperature in a sterile ventilated hood. Following therapeutic agent incubation, the wells were subsequently. . .

DETD **Vascular smooth muscle** (BO54) cells were seeded and processed in 24 well plates, as described above under "5 Minute Exposure: Protein Synthesis Assay.". . .

DETD **Vascular smooth muscle** (BO54) cells at 20,000 cells/ml were seeded in sterile 24 well plates and incubated in complete medium (1 ml/well) overnight. . .

DETD **Vascular Smooth Muscle** Cell Migration Inhibition

DETD **Vascular smooth muscle** cells (BO54) were

derived from explants of baboon aortic smooth muscle, as described in Example 10. The cells were grown in flat bottom, six well tissue culture plates, which hold about 5 ml of medium. The **vascular smooth muscle** cells were plated at 200,000 cells/well and placed at 37.degree. C. in a humidified 5% CO.sub.2 incubator for 18 hours.. . .

DETD . . . "M" indicates Migration Grade, wherein-=no migration; +1=minimal; +2=mild; +3=moderate; and +4=marked (maximum density; limit of cell contact inhibition) migration of **vascular smooth muscle** cells into the cleared area adjacent to the scratch. In this Table, "T" denotes a morphological Toxicity Grade, wherein-=no toxicity;. . .

DETD The data indicate that cytochalasin B inhibits the migration (+1 to +2) of **vascular smooth muscle** cells into the cleared area adjacent to the scratch at a dose of 0.1 .mu.g/ml with only minimal (- to. . .

DETD TABLE 4

SCRATCH-MIGRATION ASSAY: INHIBITION OF **VASCULAR SMOOTH MUSCLE** CELL MIGRATION BY CYTOCHALASIN B

Continuous Exposure				7-day Recovery Post Exposure	
Dosage .mu.g/mL				Dosage .mu.g/mL	
	Control				Control
Day	0.0	0.01	0.1	1.0	0.0. . .

DETD **Vascular smooth muscle** cells were exposed to a therapeutic agent in one of two exposure formats:

DETD In Vivo BRDU Assay: Inhibition of **Vascular Smooth Muscle**

DETD In vivo **vascular smooth muscle** proliferation was quantitated by measuring incorporation of the base analog 5-bromo-2'-deoxyuridine (BRDU, available from Sigma Chemical Co.) into DNA during. . .

DETD . . . uptake relative to a PBS control; however, cytochalasin B and staurosporin inhibited BRDU uptake (i.e., cell proliferation) without killing the **vascular smooth muscle** cells. The number of **vascular smooth muscle** cells labeled with BRDU was assigned a grade at 400.times.magnification as follows:

DETD . . . from 0.1 .mu.g/ml (.apprxeq.140% increase) to 1.0 .mu.g/ml (FIG. 14). The 10 .mu.g/ml dose appeared to be toxic to the **vascular smooth muscle** cells (data not shown). The subthreshold dose (0.01 .mu.g/ml) and negative control (PBS) exhibited a .+-..apprxeq.20% change in luminal area.. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation

DETD Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely

abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614.+-.1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta

and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-betal mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

CLM What is claimed is:

10. The method of claim 1 wherein the amount is effective to inhibit proliferation of **vascular smooth muscle** cells.

14. The method of claim 13 wherein the amount does not eliminate the ability of **vascular smooth muscle** cells to secrete extracellular matrix.

L7 ANSWER 13 OF 19 CAPLUS COPYRIGHT 2001 ACS

AN 1998:224014 CAPLUS

DN 128:317383

TI Effect of 17.beta.-estradiol on cytokine-induced nitric oxide production in rat isolated aorta

AU Kauser, Katalin; Sonnenberg, Dagmar; Diel, Patrick; Rubanyi, Gabor M.

CS Cardiovascular Department, Berlex Biosciences, Richmond, CA, 94804-0099, USA

SO Br. J. Pharmacol. (1998), 123(6), 1089-1096
CODEN: BJPCBM; ISSN: 0007-1188

PB Stockton Press

DT Journal

LA English

AB Studies were performed on isolated aortic rings without **endothelium** to investigate the effect of 17.beta.-estradiol on cytokine-induced nitric oxide prodn. by the inducible nitric oxide synthase (iNOS). Treatment of the isolated aortic rings with interleukin-1.beta. (IL-1.beta., 20 .mu. ml-1) led to the expression of iNOS mRNA and protein, as well as significant nitrite accumulation in the incubation media and suppression of phenylephrine (1 nM - 10 .mu.M)-evoked contraction. Cycloheximide (1 .mu.M), a protein synthesis inhibitor, prevented iNOS protein expression, nitrite accumulation and the suppression of contractility by IL-1.beta. on the isolated aortic rings. 17.beta.-Estradiol (1 nM - 10 .mu.M) and the partial estrogen receptor agonist 4-OH-**tamoxifen** (1 nM - 10 .mu.M) produced concn.-dependent inhibition of IL-1.beta.-induced nitrite accumulation and restored vasoconstrictor responsiveness to phenylephrine, similar to the iNOS inhibitor aminoguanidine (100 .mu.M). Semiquant. PCR demonstrated decreased iNOS mRNA in the IL-1.beta.-induced and 17.beta.-estradiol-treated rings. Western blot anal. of rat aorta homogenates revealed that 17.beta.-estradiol treatment resulted in a redn. in IL-1.beta.-induced iNOS protein level. Incubation with tumor necrosis factor .alpha. (TNF.alpha., 1 ng ml-1) resulted in significant nitrite accumulation in

the incubation media and suppression of the smooth muscle contractile response to phenylephrine, similar to IL-1.beta.. The effects of TNF.alpha. were also inhibited by co-incubation of the rings with 17.beta.-estradiol and 4-OH-**tamoxifen** (1 .mu.M). The anti-transforming growth factor-.beta.1 (TGF-.beta.1) antibody, which inhibited TGF-.beta.1-induced suppression of nitrite prodn. from IL-1.beta.-treated vascular rings, did not affect the inhibitory action of 17.beta.-estradiol, suggesting that the effect of estrogen on iNOS inhibition was not mediated by TGF-.beta.1. These results show that the ovarian sex steroid, 17.beta.-estradiol is a modulator of cytokine-induced iNOS activity in rat **vascular smooth muscle** and its mechanism of action involves decrease of iNOS mRNA and protein.

IT Aorta
Vascular smooth muscle
Vasoconstriction
(effect of 17.beta.-estradiol on cytokine-induced nitric oxide prodn. in rat isolated aorta in relation to decreased iNOS mRNA and protein)

L7 ANSWER 14 OF 19 CAPLUS COPYRIGHT 2001 ACS
AN 1998:487695 CAPLUS
DN 129:240070
TI Effects of gonadal steroids and their antagonists on DNA synthesis in human vascular cells
AU Somjen, Dalia; Kohen, Fortune; Jaffe, Anat; Amir-Zaltsman, Yehudit; Knoll, Esther; Stern, Naftali
CS Institute of Endocrinology, Tel Aviv Sourasky Medical Center, The Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv-Jaffa, 64239, Israel
SO Hypertension (1998), 32(1), 39-45
CODEN: HPRTDN; ISSN: 0194-911X
PB Williams & Wilkins
DT Journal
LA English
AB The cardiovascular effect of estrogen is currently under intense investigation, but the role of androgens in vascular biol. has attracted little attention. Because **endothelial** repair and **vascular smooth muscle** cell (VSMC) proliferation affect atherogenesis, the authors analyzed the effects of 17.beta.-estradiol (E2), dihydrotestosterone (DHT), and sex hormone antagonists on DNA synthesis in human umbilical VSMCs and in E304 cells (a human umbilical **endothelial** cell line). In VSMCs, both E2 and DHT had a biphasic effect on [3H]thymidine incorporation into DNA: low concns. (0.3 nmol/L for E2, 3 nmol/L for DHT) stimulated [3H]thymidine incorporation (+35% and +41%, resp.), whereas high concns. (30 nmol/L for E2, 300 nmol/L for DHT) inhibited [3H]thymidine incorporation (-40%). In contrast, E2 (0.3 to 300 nmol/L) and DHT (3 to 3000 nmol/L) dose-dependently enhanced [3H]thymidine incorporation in E304 cells (peak, +85% for both). In VSMCs, high concns. of E2 and DHT inhibited platelet-derived growth factor (PDGF)-or insulin-like growth factor (IGF-1)-induced DNA synthesis (-50% to 80%), whereas PDGF- or IGF-1-dependent DNA synthesis in E304 cells was further increased by E2. The antiestrogens **tamoxifen** and raloxifene mimicked the effects of E2 on DNA synthesis in both VSMCs and E304 cells. However, when coincubated with a stimulatory concn. of E2 (0.3 nmol/L), **tamoxifen** and raloxifene blocked E2-induced [3H]thymidine incorporation in E304 cells but not in VSMCs. Finally, the androgen antagonist flutamide inhibited the biphasic effects of DHT on VSMCs and blocked the increase in DNA elicited by DHT in E304 cells. The results suggest complex, dose-dependent, and cell-specific interactions of estrogens, androgens, and their resp. antagonists in the control of cellular proliferation in the vascular wall. Gonadal steroid-dependent inhibition of VSMC proliferation and stimulation of **endothelial**

replication may contribute to vascular protection and remodeling responses
to vascular injury.
IT Cell proliferation
DNA formation
Vascular smooth muscle
(effects of gonadal steroids and their antagonists on DNA synthesis in human vascular cells)

L7 ANSWER 15 OF 19 USPATFULL
AN 97:5708 USPATFULL
TI Method for identifying an agent which increases TGF-beta levels
IN Grainger, David J., Cambridge, England
Metcalfe, James C., Cambridge, England
PA NeoRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 5595722 19970121
AI US 1995-476735 19950607 (8)
RLI Continuation-in-part of Ser. No. US 1994-242161, filed on 12 May 1994 which is a continuation-in-part of Ser. No. US 1993-61714, filed on 13 May 1993, now abandoned And Ser. No. US 1994-241844, filed on 12 May 1994 which is a continuation-in-part of Ser. No. US 1993-62451, filed on
13 May 1993, now abandoned which is a continuation-in-part of Ser. No. US 1993-11669, filed on 28 Jan 1993, now abandoned
DT Utility
EXNAM Primary Examiner: Henley, III, Raymond
LREP Schwegman, Lundberg, Woessner & Kluth, P.A.
CLMN Number of Claims: 7
ECL Exemplary Claim: 1
DRWN 2 Drawing Figure(s); 2 Drawing Page(s)
LN.CNT 4090
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
SUMM . . . can be considered to occur in five overlapping stages such as migration, lipid accumulation, recruitment of inflammatory cells, proliferation of **vascular smooth muscle** cells, and extracellular matrix deposition. Each of these processes can be shown to occur in man and in animal models. . .
SUMM The administered compound of formula (I) can act on **vascular smooth muscle** cells (VSMC) to inhibit the pathological activity of these smooth muscle cells and can inhibit lipid proliferative lesions. Preferably, the. . . comprises treatment of atherosclerosis, wherein the compound of formula (I), such as idoxifene or idoxifene salt, inhibits lipid accumulation by **vascular smooth muscle** cells and/or stabilizes an arterial lesion associated with atherosclerosis, i.e., increases plaque stability, to prevent rupture or growth of the. . .
SUMM A further aspect of the invention is a method comprising inhibiting **vascular smooth muscle** cell proliferation associated with procedural vascular trauma due to organ transplantation, vascular surgery, angioplasty, shunt placement, stent placement or vascular. . .
SUMM Yet a further aspect of the invention provides a method comprising inhibiting non-aortal **vascular smooth muscle** cell proliferation associated with procedural vascular trauma comprising administering an effective cytostatic antiproliferative amount of tamoxifen or a structural analog. . .
SUMM . . . of the present invention is a method for identifying a compound which is a TGF-beta activator or production stimulator. Human **vascular smooth muscle** cells (hVSMC) are cultured with an amount of the compound effective to reduce the normal rate of hVSMC proliferation, due. . . with an amount of an antibody which neutralizes TGF-beta activity. The method can also include the culture of rat aortic **vascular smooth muscle**

cells (rVSMC) with an amount of the same compound effective to reduce the normal rate of proliferation of rVSMC, due. . .

SUMM . . . in the practice of the present invention to prevent or treat other conditions characterized by inappropriate or pathological activity of **vascular smooth muscle** cells. Such TGF-beta activators and production stimulators inhibit abnormal activity of **vascular smooth muscle** cells. Preferred compounds of formula (I) include those wherein Z is a covalent bond, Y is H, R.sup.3 is ClCH.sub.2. . .

DRWD FIGS. 1 and 2 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.

DETD . . . with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a **vascular smooth muscle** cell or pericyte.

DETD . . . thereof, which are capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell activity. Isomers and derivatives of the aforementioned chemical compound are also included within the scope of the term "tamoxifen". . .

DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta.

DETD TGF-beta activators or production stimulators of the invention are useful for inhibiting the pathological proliferation of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .

DETD . . . a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle** cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 1. TGF-beta is believed to contribute to the inhibitory mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels.

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. An apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 2. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad.

Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82.+-.4 hours to 47.+-.4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7.+-.0.6 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47.+-.3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82.+-.4 hours. . . doubling time for cultures of this experiment being 45.+-.6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells

completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 (a concentration resulting in 50% inhibition) at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42.+-.2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 1 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 2 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that are. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD . . . dosage forms involving sustained release of the TGF-beta activator or production stimulator to target cells. Preferably, the target cells are **vascular smooth muscle** cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases

that. . . the dosage form. Consequently, the methods and dosage forms
of this aspect of the present invention are useful for inhibiting **vascular smooth muscle** cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, formation of lipid proliferative lesions, contraction, migration or the like) but does not kill the cell and, optionally, a **vascular smooth muscle** cell binding protein. Sustained released dosage forms for systemic administration as well as for local administration are also employed in.

. .

DETD . . . affect the rate and duration of the drug release required to achieve the cytostatic dosing which has been demonstrated in **vascular smooth muscle** cell tissue culture experiments. Different types of devices may require different periods of
therapeutic drug release. For example, the use. . .

DETD Human **vascular smooth muscle** cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat. Medium conditioned. . .

DETD . . . is a TGF-beta activator or TGF-beta production stimulator, an agent or mixture of agents is first tested on rat aortic **vascular smooth muscle** cells (rVSMCs) for their ability to stimulate the production of active TGF-.beta. in the culture medium as originally described for. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship thereof to TGF-Beta Production and Activation

DETD Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all of the cells. To
distinguish between these possibilities,. . .

DETD Quiescent **vascular smooth muscle** cells were stimulated with DMEM+10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being. . . at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the **vascular smooth muscle** cells had divided by 40 hours, whereas there was no significant increase in cell number in the presence of tamoxifen. . . micromolar tamoxifen. Since tamoxifen did not significantly reduce the

proportion of cells completing the cell cycle and dividing, inhibition of **vascular smooth muscle** cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all. . .

DETD To determine whether tamoxifen increased the duration of the cell cycle of **vascular smooth muscle** cells by increasing the duration of the G.sub.0 to S phase, the effect of tamoxifen on entry into DNA synthesis. . . did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent **vascular smooth muscle** cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [.sup.3 H]-thymidine incorporation: control at 17614.+-.1714. . . time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured **vascular smooth muscle** cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987. Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity. When quiescent **vascular smooth muscle** cells were stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta,. . .

DETD To confirm that the **vascular smooth muscle** cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of. . .

DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from <5% in the medium conditioned on **vascular smooth muscle** cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD Heparin increases TGF-beta activity in medium conditioned on **vascular smooth muscle** cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from. . .

DETD . . . content of TGF-beta mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat **vascular smooth muscle** cells (6th passage in exponential growth) in the absence or presence of ethanol vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate

orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. **Tamoxifen** (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were purchased from Flow Laboratories. 6-[.sup.3. . . factor and insulin-like growth factor 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Antiotensin II and **endothelin** 1 were obtained from Sigma and dissolved in sterile MilliQ water. TGF-beta (0.5 .mu.g, lyophilized solid) was purchased from Peninsula,. . .

DETD TABLE 8

Mitogenic indices of human serum and plasma on human **vascular smooth muscle** cells

Donor	Mitogenic index Serum	Plasma
B	45	0.7
H	52	1.4
C	60	0.9
D	65	1.0
A	83	1.2

DMEM containing 5% serum or. . .

L7 ANSWER 16 OF 19 CAPLUS COPYRIGHT 2001 ACS
AN 1997:440538 CAPLUS
DN 127:131167
TI Estrogen relaxation of coronary artery smooth muscle is mediated by nitric oxide and cGMP
AU Darkow, David J.; Lu, Luo; White, Richard E.
CS Dep. Physiology and Biiophysics, Wright State Univ. Sch. Medicine, Dayton, OH, 45435, USA
SO Am. J. Physiol. (1997), 272(6, Pt. 2), H2765-H2773
CODEN: AJPHAP; ISSN: 0002-9513
PB American Physiological Society
DT Journal
LA English
AB Estrogens are proposed to exert protection against cardiovascular disease, and evidence now suggests that this protection involves a direct vasodilatory effect. We have shown previously that estrogen relaxes **endothelium**-denuded porcine coronary arteries by opening the large-conductance calcium- and voltage-activated potassium (BKCa) channel of myocytes through cGMP-dependent phosphorylation. The present study confirms these results and now demonstrates that this mechanism involves prodn. of nitric oxide (NO). S-nitroso-N-acetylpenicillamine (SNAP), an NO donor, or 8-bromo-cGMP mimicked the effect of estrogen on BKCa channels. Furthermore inhibition of NO synthase (NOS) attenuated estrogen- or **tamoxifen**-induced BKCa-channel activity, and this effect was disinhibited by L-arginine. Inhibition of guanyl cyclase activity blocked the stimulatory effect of estrogen, SNAP, or L-arginine on BKCa channels. Furthermore, 17.beta.-estradiol stimulated accumulation of nitrite and cGMP in coronary myocytes. Therefore, we propose that the vasodilatory effect of estrogen on the coronary circulation is mediated by NO. A portion of the beneficial cardiovascular effects of estrogen may be attributed to relaxation of **vascular smooth muscle** by a process that involves NO- and cGMP-dependent stimulation of BKCa channels.
IT Coronary artery
Myocyte (heart)
Potassium transport (biological)

Signal transduction (biological)
Smooth muscle
Vascular smooth muscle
(estrogen relaxation of coronary artery smooth muscle mediation by nitric oxide and cGMP)

L7 ANSWER 17 OF 19 USPATFULL
AN 96:72817 USPATFULL
TI Prevention and treatment of pathologies associated with abnormally proliferative smooth muscle cells
IN Grainger, David J., Cambridge, United Kingdom
Metcalfe, James C., Cambridge, United Kingdom
Weissberg, Peter L., Cambridge, United Kingdom
PA NeoRx Corporation, Seattle, WA, United States (U.S. corporation)
PI US 5545569 19960813
AI US 1995-450520 19950525 (8)
RLI Division of Ser. No. US 1994-242161, filed on 12 May 1994 which is a continuation-in-part of Ser. No. US 1993-61714, filed on 13 May 1993, now abandoned
DT Utility
EXNAM Primary Examiner: Henley, III, Raymond
LREP Schwegman, Lundberg, Woessner & Kluth, P.A.
CLMN Number of Claims: 12
ECL Exemplary Claim: 1
DRWN 2 Drawing Figure(s); 2 Drawing Page(s)
LN.CNT 2263
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB . . . dose are also amenable to chronic use for prophylactic purposes
with respect to disease states involving proliferation and/or migration of **vascular smooth muscle** cells over time.
Further provided is a method for determining TGF-beta in vitro, thereby identifying a patient at risk for. . .
SUMM . . . to the prevention and treatment of conditions characterized by abnormal smooth muscle cell proliferation. More specifically, mechanisms
for in vivo **vascular smooth muscle** cell
proliferation modulation and agents that impact those mechanisms are discussed.
SUMM . . . cell proliferation. It would be highly advantageous to develop new compositions or methods for inhibiting stenosis due to proliferation
of **vascular smooth muscle** cells following,
for example, traumatic injury to vessels rendered during vascular surgery.
SUMM . . . a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of **vascular smooth muscle**
cells over time (e.g., atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma. . .
DRWD FIGS. 1 and 2 depict pathways for the modulation of **vascular smooth muscle** cell proliferation in vivo.
DETD . . . with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a **vascular smooth muscle**
cell or pericyte.
DETD . . . trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine which is capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits **vascular smooth muscle** cell
proliferation. Functional equivalents and derivatives of the aforementioned chemical compound are also included within the scope of the term. . .
DETD . . . latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of

inhibiting **vascular smooth muscle** cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta.

DETD TGF-beta activators or production stimulators of the invention are useful for inhibiting the pathological proliferation of **vascular smooth muscle** cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal. . .

DETD . . . pathogenic conditions, is the proliferation or the migration of smooth muscle cells. No direct link between Lp(a) and proliferation of **vascular smooth muscle** cells had been established in the prior art.

DETD An in vivo pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 1. This mechanism is believed to constitute a portion of the mechanism that maintains **vascular smooth muscle** cells in a non-proliferative state in healthy vessels.

DETD **Vascular smooth muscle** cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example. . . TGF-beta from inactive compleyes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of **vascular smooth muscle** cells. The apparent in vivo physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through. . . the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of **vascular smooth muscle** cells.

DETD An additional pathway for the modulation of **vascular smooth muscle** cell proliferation is shown in FIG. 2. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such. . .

DETD . . . activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci, USA, 86: 3847, 1989). Experiments conducted on human aortic **vascular smooth muscle** cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM)+10% fetal calf serum (FCS) as described. . .

DETD 1) Addition of Lp(a) to sub-confluent human **vascular smooth muscle** cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human **vascular smooth muscle** cells caused a reduction in doubling time from 82+/-4 hours to 47+/-4 hours);

DETD . . . acid treatment or in vivo by the serine protease plasmin) in order to become capable of inhibiting the proliferation of **vascular smooth muscle** cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

DETD The hypothesis that Lp(a) and apo(a) were acting on cultured human **vascular smooth muscle** cells by interfering with activation of latent TGF-beta was tested. In support of this hypothesis, an observation was made that plasmin activity associated with **vascular smooth muscle** cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in **vascular smooth muscle** cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than. . .

DETD . . . the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human **vascular smooth muscle** cell cultures were measured in the presence and absence

of the lipoproteins and in the presence of a large excess. . . as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the **vascular smooth muscle** cell cultures. For example, plasminogen activator activity in the conditioned medium remained at 0.7+/-0.6 mU/ml with Lp(a) additions up to. . .

DETD . . . by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human **vascular smooth muscle** cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

DETD . . . conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human **vascular smooth muscle** cells were cultured in the presence of Lp(a). These cells had a population doubling time of 47+/-3 hours. Addition of. . .

DETD . . . role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human **vascular smooth muscle** cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82+/-4 hours. . . doubling time for cultures of this experiment being 45+/-6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in **vascular smooth muscle** cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of **vascular smooth muscle** cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of. . .

DETD Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and **vascular smooth muscle** cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

DETD 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of **vascular smooth muscle** cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly. . .

DETD 3) Tamoxifen decreases the rate of proliferation of serum-stimulated **vascular smooth muscle** cells by increasing the time taken to traverse the G.sub.2 to M phase of the cell cycle.

DETD 4) Tamoxifen decreased the rate of proliferation of **vascular smooth muscle** cells by inducing TGF-beta activity.

DETD 5) **Vascular smooth muscle** cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat **vascular smooth muscle** cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

DETD 8) Tamoxifen is a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 (a concentration resulting in 50% inhibition) at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat **vascular smooth muscle** cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the presence of tamoxifen and Lp(a) being 42+/-2. . . levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat **vascular smooth muscle** cells treated with tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 1 can be identified by a practitioner in the art by conducting. . .

DETD Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit **vascular smooth muscle** cell proliferation by the pathway shown in FIG. 2 can be identified by a practitioner in the art by conducting. . .

DETD . . . and the like, having at least one of the activities recited above and therefore being capable of inhibiting proliferation of **vascular smooth muscle** cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for **vascular smooth muscle** cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and. . .

DETD . . . specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of **vascular smooth muscle** cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against atherosclerosis and subsequent myocardial infarctions that are. . . activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for **vascular smooth muscle** cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued lower dose delivery to the traumatized site further protects against restenosis resulting from **vascular smooth muscle** cell proliferation in the traumatized area.

DETD Human **vascular smooth muscle** cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat (doubling time. . .

DETD Impact of Tamoxifen on **Vascular Smooth Muscle** Cells and the Relationship Thereof to TGF-Beta Production and Activation

DETD Cell culture, DNA synthesis assay and cell counting. Rat **vascular smooth muscle** cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger. . .

DETD . . . cells/cm.sup.2 on tissue culture plastic. When the cells reached confluence (after about 10 days), they were subcultured as described for **vascular smooth muscle** cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DETD DNA synthesis was assayed by [.sup.3 H]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. **Vascular smooth muscle** cells were subcultured, grown in DMEM+10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated. . .

DETD . . . Bedford, Mass.). At 10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) **vascular smooth muscle** cells.

DETD RNA Preparation and Northern Analysis, Total cytoplasmic RNA was isolated from cultured **vascular smooth muscle** cells as described in Kemp et al., Biochem. J., 277: 285-288, 1991. Northern analysis was performed by electrophoresis of total. . .

DETD Results, **Vascular smooth muscle** cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM+10% FCS. . . The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the **vascular smooth muscle** cells or from an increase in the cell cycle time of all

of the cells. To distinguish between these possibilities,. . .
DETD Quiescent **vascular smooth muscle** cells
were stimulated with DMEM +10% FCS in the absence or presence of 33
micromolar tamoxifen, with the cell number. . . at 8 hour intervals
by time lapse photomicroscopy. In the presence of ethanol vehicle
alone,
more than 95% of the **vascular smooth muscle**
cells had divided by 40 hours, whereas there was no significant
increase
in cell number in the presence of tamoxifen. . . micromolar
tamoxifen. Since tamoxifen did not significantly reduce the proportion
of cells completing the cell cycle and dividing, inhibition of
vascular smooth muscle cells caused by
tamoxifen appears to be the result of an increase in the cell cycle
time
of nearly all. . .
DETD To determine whether tamoxifen increased the duration of the cell cycle
of **vascular smooth muscle** cells by
increasing the duration of the G.sub.0 to S phase, the effect of
tamoxifen on entry into DNA synthesis. . . did not significantly
affect the time course or the proportion of cells entering DNA
synthesis
following serum stimulation of quiescent **vascular**
smooth muscle cells (DNA synthesis between 12 hours
and 24 hours after stimulation was measured by [.sup.3 H]-thymidine
incorporation: control at 17614+/-1714. . . time course of entry
into
DNA synthesis. These results therefore imply that tamoxifen decreases
the rate of proliferation of serum-stimulated **vascular**
smooth muscle cells by increasing the time taken to
traverse the G.sub.2 to M phase of the cell cycle.
DETD . . . for TGF-beta (see, for example, Assoian et al., J. Cell,
Biol.,
109: 441-448, 1986) with respect to proliferation of subcultured
vascular smooth muscle cells in the presence
of serum. Tamoxifen is known to induce TGF-beta activity in cultures of
breast carcinoma cell lines. . . et al., Cell, 48: 417-425, 1987.
Consequently, experimentation was conducted to determine whether
tamoxifen decreased the rate of proliferation of **vascular**
smooth muscle cells by inducing TGF-beta activity.
When quiescent **vascular smooth muscle**
cells were stimulated with 10% FCS in the presence of 50 micromolar
tamoxifen and 10 micrograms/ml neutralizing antiserum against
TGF-beta,.
. .
DETD To confirm that the **vascular smooth muscle**
cells produced TGF-beta in response to tamoxifen, such cells were
treated with tamoxifen for 96 hours in the presence of. . .
DETD . . . 4-fold. Furthermore, the proportion of the TGF-beta present in
active form was increased from <5% in the medium conditioned on
vascular smooth muscle cells in the presence
of ethanol vehicle alone to approximately 35% in the medium conditioned
on cells treated with tamoxifen. Thus, tamoxifen appears to increase
TGF-beta activity in cultures of rat **vascular smooth**
muscle cells by stimulating the production of latent TGF-beta
and increasing the proportion of the total TGF-beta which has been
activated.
DETD Heparin increases TGF-beta activity in medium conditioned on
vascular smooth muscle cells (unpublished
data). The mechanism of action of heparin in this regard appears to
involve the release of TGF-beta from. . .
DETD . . . content of TGF-beta1 mRNA was also analyzed by Northern
analysis at various time points after addition of tamoxifen.
Subcultured
rat **vascular smooth muscle** cells (6th
passage in exponential growth) in the absence or presence of ethanol

vehicle alone contain very little mRNA for. . .

DETD Although TGF-beta decreases the rate of proliferation of **vascular smooth muscle** cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of **vascular smooth muscle** proliferation with an ED.sub.50 at least 10-fold lower for **vascular smooth muscle** cells than for adventitial fibroblasts.

DETD Materials. Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. **Tamoxifen** (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were purchased from Flow Laboratories. 6-[.sup.3. . . factor and insulin-like growth facter 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Antiotensin II and **endothelin** 1 were obtained from Sigma and dissolved in sterile MilliQ water. TGF-beta (0.5 .mu.g, lyophilized solid) was purchased from Peninsula,. . .

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AN 1996:234315 CAPLUS

DN 124:279469

TI 17.beta.-Estradiol and smooth muscle cell proliferation in aortic cells of male and female rats

AU Espinosa, Emma; Oemar, Barry S.; Luescher, Thomas F.

CS Cardiovascular Res., Univ. Hosp., Bern, CH-3010, Switz.

SO Biochem. Biophys. Res. Commun. (1996), 221(1), 8-14
CODEN: BBRCA9; ISSN: 0006-291X

DT Journal

LA English

AB The low incidence of cardiovascular disease in women before menopause or during hormone replacement therapy suggests a protective effect of estrogens. The mechanism(s) are uncertain but may involve effects on lipids, coagulation and the **endothelium**. **Vascular smooth muscle** cell (VSMC) proliferation also contributes to atherosclerosis. Hence, we investigated whether 17.beta.-estradiol (E2) inhibits VSMC proliferation. VSMC of 6 female and 6 male Wistar Kyoto rats (WKY; age 10-12 wk) were incubated for 24 h with E2 and/or fetal calf serum (FCS). E2 (10-9-10-5 M) alone reduced [3H]thymidine uptake at 10-5 M in female cells only. In female and male VSMC, FCS (1%) increased [3H]thymidine uptake (4.5-fold). When given simultaneously, E2 did not prevent this effect of FCS (1%). However, when cells were preincubated for 24 h with E2 and then stimulated with FCS, [3H]thymidine uptake was reduced by E2 at 10-5 M in female VSMC, while in male VSMC this effect was minimal. Both female and male VSMC expressed estrogen receptors as demonstrated by RT-PCR. Pretreatment of female VSMC cells with the E2 receptor antagonist **tamoxifen** prevented the antiproliferative effects exerted by E2. In aortic VSMC of female rats, E2 moderately inhibited proliferation on its own and during stimulation with FCS, while this effect was small in VSMC of male rats. The expression of the E2 receptor in female and male VSMC and the effects of **tamoxifen** suggest that this effect is mediated by E2 receptors.

L7 ANSWER 19 OF 19 CAPLUS COPYRIGHT 2001 ACS

AN 1993:1051 CAPLUS

DN 118:1051

TI In vitro effect of estradiol on thymidine uptake in pulmonary **vascular smooth muscle** cell: role of the endothelium

AU Farhat, Michel Y.; Vargas, Roberto; Dingaan, Brenda; Ramwell, Peter W.

CS Med. Cent., Georgetown Univ., Washington, DC, 20007, USA

SO Br. J. Pharmacol. (1992), 107(3), 679-83
CODEN: BJPCBM; ISSN: 0007-1188
DT Journal
LA English
TI In vitro effect of estradiol on thymidine uptake in pulmonary **vascular smooth muscle** cell: role of the endothelium
AB The effect of different concns. of 17.beta.-estradiol (3-300 nM) on [3H]thymidine uptake was studied in segments from canine pulmonary artery, and cultures of rat pulmonary **vascular smooth muscle** cells (VSMC). Incubation with estradiol for 24 h, potentiated in a concn.-dependent manner [3H]thymidine uptake in VSMC cultures. Estradiol potentiated thymidine uptake by pulmonary arterial segments but only when the **endothelium** had been removed. Autoradiog. showed dense incorporation of radioactive thymidine in the **vascular smooth muscle** cells of the media. The nonsteroidal estrogen, stilbestrol (300 nM), also significantly potentiated [3H]thymidine uptake, in both VSMC cultures and pulmonary artery segments. Testosterone was ineffective at a similar concn. Preincubation of the pulmonary VSMC with the antiestrogen **tamoxifen** (1 .mu.M) antagonized the potentiating effect of estradiol on [3H]thymidine incorporation. The effect of **tamoxifen** was less pronounced in pulmonary arterial segments. Thus, estrogen may promote proliferation of pulmonary VSMC. **Endothelial** injury or dysfunction may be an important factor in the expression of the estrogenic effect. Moreover, plasma estrogen may be a contributing factor to the proliferative lesion obsd. in certain forms of pulmonary vascular injury in women.
IT Cell proliferation
(of pulmonary **vascular smooth muscle** cells, estrogen induction of, endothelium role in)
IT Lung
(proliferation of **vascular smooth muscle** cells of, estrogen effect on, endothelium role in)
IT Estrogens
RL: BIOL (Biological study)
(pulmonary **vascular smooth muscle** cell proliferation response to, endothelium role in)
IT Receptors
RL: BIOL (Biological study)
(estrogen, pulmonary **vascular smooth muscle** cell proliferation regulation by)
IT Estrogens
RL: BIOL (Biological study)
(receptors, pulmonary **vascular smooth muscle** cell proliferation regulation by)
IT 50-28-2, Estradiol, biological studies 56-53-1, Stilbestrol
RL: BIOL (Biological study)
(pulmonary **vascular smooth muscle** cell proliferation response to, endothelium role in)